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MORPHOLOGICAL AND BIOCHEMICAL STUDIES ON THE CHORIO-ALLANTOIS OF THE CHICK EMBRYO FOLLOWING INFECTION WITH CERTAIN VIRUSES.*

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NUMEROUS studies on the metabolism of tissues infected with viruses have shown that various detectable biochemical changes result from virus infection (*cf.* the excellent review of the subject by Bauer, 1953). Preliminary work in this laboratory demonstrated that the chorio-allantoic membrane of the chick embryo, when infected with myxoma virus, displays a marked increase in its ability to form lactic acid from glucose without any concomitant impairment of respiration, indicating that it is due to an increase in glycolysis. The increased glycolysis depends in turn upon the greater rate at which fructose-1-6-diphosphate (HDP) is split to triose phosphates. This reaction is catalysed by zymohexase (aldolase) and determinations of this enzyme were therefore done on extracts of myxoma-infected chorio-allantoic membranes; these extracts did, indeed, display far greater zymohexase activity than extracts of normal membranes similarly prepared.

The above observations led to a more intensive study of glycolysis in the chorio-allantoic membrane and its relation to virus infection. We have attempted to relate biochemical effects to the histologic changes observable at various time intervals in the infected membrane and have investigated the effect of various viruses on the membrane, with a view to answering the following questions:

1. Does the increase in glycolysis depend upon some specific pathologic change in the membrane, such as ectodermal hyperplasia, or inflammation, or necrosis, or does it depend upon viral multiplication?

2. Is the increased rate of glycolysis characteristic of infections with myxoma virus only, or does it occur when the membrane is infected with other viruses?

3. Must the myxoma (or other) virus be infective to increase glycolysis, or will heat-inactivated virus exert the same effect?

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The experimental data to be presented support the view that the increased rate of glycolysis is related to viral multiplication rather than to the cellular response elicited in the membrane by infection or trauma.

MATERIALS AND METHODS.

Embryonated eggs.

Eggs from Rhode Island Red hens were obtained from the same local hatchery throughout the study. They were inoculated on the 11th day of incubation (unless otherwise stated) after preliminary dropping of the membrane, then incubated at 35°. In every experiment eggs of the same batch, inoculated with a suspension of lyophilized normal membrane or else with sterile broth or saline, served as controls.

Viruses.

Myxoma virus was obtained from Dr. R. E. Shope, in whose laboratory at the Rockefeller Institute it had been passed in rabbits for some years. It had subsequently undergone 39 passages on the chorio-allantoic membrane. The method of preparing and inoculating the virus was described in an earlier paper (Kun and Smith, 1950).

Fibroma virus (Boerlage strain) was also obtained from Dr. R. E. Shope. Isolated in 1947 from a wild cottontail rabbit in New Jersey, this strain, like the OA strain, can readily be adapted to growth on the chorio-allantoic membrane (Smith, 1948).

Rous sarcoma virus was obtained from Dr. K. Porter of the Rockefeller Institute and passed several times in eggs before use ; the lesions were in every way like those described by Keogh (1938).

Herpes simplex virus was furnished by Dr. G. J. Buddingh, who isolated it from a child ill with herpetic stomatitis. It had undergone many egg passages in his laboratory and produced chorio-allantoic lesions as described by Anderson (1940).

Vaccinia virus, a dermal strain obtained from Dr. G. J. Buddingh, produced lesions comparable to those described by Goodpasture, Woodruff and Buddingh (1932).

Egg-adapted *Newcastle disease* virus, strain B, was obtained from Dr. G. J. Buddingh.

Mouse-adapted *Swine influenza* virus, strain V15, obtained from Dr. R. E. Shope, was subjected to 9 passages in the allantoic sac before use in these experiments.

Virus titration.

Titrations were carried out on every pool of chorio-allantoic membranes subjected to metabolic studies. In the case of myxoma, vaccinia, herpes and Rous sarcoma viruses, infectivity titrations were carried out by the pock-counting method (Beveridge and Burnet, 1946). Fibroma virus was titrated in the skin of a rabbit and the titres of Newcastle and influenza viruses were determined by haemagglutination.

Celite.

Sterile inflammation was induced by the use of a suspension of Celite*, containing between 0·1 and 0·5 g. in 10 ml. of 0·9 per cent NaCl solution, sterilized by autoclaving and inoculated onto the membrane in the same way as the viruses. This material was employed, rather than diatomaceous earth or other silica compounds, simply because it was readily available in our laboratory.

Sterility.

Portions of each membrane used for metabolic determinations were checked for sterility by cultivation in fluid thioglycollate medium.† The data from the one experiment involving membranes contaminated with bacteria are excluded. It must, however, be stated that whenever bacterial contamination occurs, the whole picture is entirely obscured ; thus sterility is a decisive criterion in all virus experiments.

* The Celite was kindly supplied by Mr. Scott Robbins of the Johns-Manville Company, New Orleans, Louisiana. He gave the following percentages of constituents : 90·9 SiO_2 ; 3·7 Al_2O_3 ; 0·1 TiO_2 ; 0·2 CaO ; 0·7 MgO ; 2·5 Na_2O ; 1·5 Te_2O_3.

† Baltimore Biological Laboratory, Incorporated.

Histologic techniques.

Portions of the same membranes used for metabolic determinations were fixed in Bouin's solution for 3 hr., then washed repeatedly in 80 per cent alcohol before embedding in paraffin. Sections were stained with phloxin methylene blue and examined microscopically under oil immersion.

Chemical methods.

All determinations were carried out on a pool of 4–8 membranes which had been similarly inoculated. The measurement of oxygen uptake and aerobic lactic acid formation by intact chorio-allantoic membranes was carried out as described previously (Kun and Smith, 1950). The membranes were suspended in Krebs-Henseleit solution containing glucose at a final concentration of 10^{-2} M. The gas phase was 100 per cent O_2. The CO_2 produced during the experiment was absorbed by 0·1 ml. of 20 per cent KOH solution which was placed in the centre well together with a strip of filter paper. The results were expressed as Q_{O_2} (μl. O_2 absorbed in 1 hr./mg. dry wt.) and $Q_{\text{lactic acid}}$ (μg. lactic acid produced in 1 hr./mg. dry wt.).

The anaerobic dismutation of fructose-1-6-diphosphate was determined in the manometric system of Kennedy and Lehninger (1949). A weighed amount (150–250 mg.) of chorio-allantoic membrane was quickly ground in an all-glass homogenizer with 3–5 ml. of ice-cold glass-distilled water. Of homogenate 0·5–1·0 ml. was pipetted together with the other constituents of the test system into the main compartment of the respirometer flasks. The vessels were flushed for 5 min. with a mixture of 95 per cent N_2 and 5 per cent CO_2, then equilibrated for 6 min. at 37°. The reaction was started by tipping in a solution of 3 μmol. (in 0·2 ml.) of neutralized coenzyme I (95 per cent purity, obtained from Sigma Chemical Company, St. Louis, Missouri) from the sidearm. The rate of CO_2 production was linear with time in the first 40–50 min. and directly proportional to the amount of homogenate. The rate of acid formation from hexose diphosphate was calculated from the first 20–25 min. readings and expressed as Q_{CO_2} (μl. CO_2 produced with HDP as substrate in 1 hr./mg. dry wt. of the membrane).

Zymohexase activity was measured by the colorimetric procedure of Sibley and Lehninger (1949). A weighed amount of the chorio-allantoic membrane was homogenized in ice-chilled 0·15 M KCl and the homogenate spun at 10,000 *g* for 10 min. at 4°. One ml. of the supernatant was incubated for 15 min. at 37° in the test system of Sibley and Lehninger, and the triose chromogen developed exactly as described by them. Since there is a linear relationship between the absorbancy of the triose chromogen (measured at 540 mμ.) and enzyme activity, it was felt that sufficiently accurate comparisons of enzyme activities could be obtained by expressing the results in terms of absorbancy (determined on the Coleman spectrophotometer) per mg. dry wt. of the membrane. The time of incubation was kept constant at 15 min. in all zymohexase assays. The dry weight of the chorio-allantoic membranes, determined in each experiment, varied between 6–18 per cent of the wet weight.

RESULTS.

Comparison of histological changes in membranes infected with various viruses and with Celite.

The chorio-allantois is so relatively simple a tissue in its organization that all lesions which it may display, whether traumatic or viral in origin, are bound to resemble each other to a considerable degree. Moreover it is difficult to compare accurately the changes produced by different viruses in the chorio-allantois, even in eggs of identical age, because the size of the inoculum has a decided influence on the character of the lesion. Nevertheless the viruses selected for study give rise to a wide variety of lesions, as we ourselves were able to observe in the course of many careful microscopic studies of infected membranes.

In the case of myxoma virus, hyperplasia of the ectoderm and migration of leucocytes into the mesoderm become apparent within five hours of inoculation, followed soon afterwards by the appearance of many small bright acidophilic

granules in the cytoplasm and enlargement of the nuclei of the ectodermal cells. Later, enormous hyperplasia of the ectoderm develops, with inflammation and capillary thromboses in the mesoderm. Finally necrosis and healing ensue and the embryo will in most cases go on to hatch if allowed to do so (Table III).

With Rous sarcoma virus infection, the lesion consists of tremendous proliferation of the ectoderm with little or no inflammatory reaction on the part of the mesoderm ; the capillaries appear normal and the endodermal layer does not seem to be involved. The lesion progresses steadily and there is no tendency to heal.

Vaccinia produces in the chorio-allantois lesions rather similar to those of myxoma except that here inflammatory changes, haemorrhage and focal hyperplasia of the capillary endothelium are more pronounced and necrosis is more marked. The infection is lethal for the embryo.

With herpes simplex infection, proliferation of ectoderm and mesoderm are accompanied by a moderate amount of inflammatory reaction, followed later by extensive necrosis and desquamation of ectodermal cells. The presence of intranuclear inclusions in the ectodermal and mesodermal cells of the membrane are the most characteristic feature of this infection in embryos inoculated on the 11th day.

Fibroma virus produces very different lesions on the chorio-allantois, depending on the strain used. Whereas the OA strain affects the membrane hardly at all (Smith, 1948), the Boerlage strain employed in these experiments gives rise to ectodermal hyperplasia with acidophilic inclusions in the cytoplasm. The mesoderm is heavily infiltrated with eosinophils, but what sets the lesions apart from any others that we have seen is the heavy infiltration of the mesoderm, especially around the blood vessels, with the long spindle-shaped cells typical of fibroma in the rabbit. Development of the embryo appears to be little affected by this infection.

The change induced by Celite is an intense inflammation of all three cell layers with widespread necrosis of both ectodermal and mesodermal tissues. With heavier suspensions of Celite (0·25–0·5 g. in 10 ml. saline) the embryo died within 48 hr. after inoculation ; with a light suspension (0·1 g. in 10 ml. saline) definite though less striking lesions occurred, and the embryo survived for several days.

Biochemical changes in membranes infected with myxoma virus.

The biochemical experiments dealing with the effect of myxoma virus on the chorio-allantoic membrane are summarized in Tables I and II. It appears that the overall rate of oxygen uptake does not change appreciably during the whole experimental period (Table I, A). Aerobic lactic acid production, on the other hand, does increase in the virus-infected membrane. The change in the rate of lactic acid production is gradual, starting from a rate lower than that of the uninfected controls and reaching its maximum 144 hr. after infection. Thereafter a gradual decline occurs to the level of the control membranes (Table I, B). A similar time relationship is observed with homogenates of chorio-allantoic membranes when the rates of acid production from HDP are compared under anaerobic conditions (Table I, C).

It is clear that the membranes infected with virus show a marked increase in aerobic lactic acid formation and that the homogenates of the virus-infected

TABLE I.—*Effect of Myxoma Virus on Respiration* (A), *Aerobic Lactic Acid Formation* (B), *and Hexose Diphosphate Dismutation* (C) *in Chorio-allantoic Membranes.*

Hours after infection.*	Virus titre.†	A. Q_{O_2}.		B. Q Lactic acid.		C. Q_{CO_2}.	
		Normal.	Infected.	Normal.	Infected.	Normal.	Infected.
12	10^{-2}	14	15	12	7	21	13
24	10^{-4}	17	16	9	8	16	14
36	10^{-6}	15	15	6	11	6	17
48	10^{-5}	16	13	12	20	7	14
72	10^{-5}	20	19	15	22	13	24
96	10^{-4}	18	15	17	29	14	32
144	10^{-4}	15	15	15	32	10	41
192	10^{-2}	20	17	18	30	8	24
288	—	18	15	17	19	12	9

Q_{O_2} = μl. oxygen absorbed in 60 min./mg. dry wt. of membrane.

Q Lactic acid = μg. lactic acid produced by membrane in 60 min./mg. dry wt. in 100 per cent O_2 atmosphere.

Q_{CO_2} = μl. CO_2 evolved in 60 min./mg. dry wt., using fructose-1-6-diphosphate as substrate under anaerobic conditions. Q_{CO_2} was determined in homogenates.

* Embryos of 8 days' incubation were used in this experiment.

† Expressed as the highest dilution at which one or more pocks were present per membrane used for the titration.

TABLE II.—*Effect of Infection with Myxoma Virus on the Zymohexase Activity of Chorio-allantoic Membrane Extracts.*

Hours after infection.*	Virus titre.	Zymohexase.†	
		Normal.	Infected.
2	10^{-2}	130	150
5	10^{-2}	145	150
8	10^{-1}	120	125
12	10^{-2}	130	125
24	10^{-4}	120	195
48	10^{-5}	105	200
67	10^{-6}	110	200
120	10^{-4}	150	235
192	10^{-3}	140	240
264	10^{-3}	125	170
288	$<10^{-3}$	150	150

* Embryos of 8 days' incubation used in this experiment.

† Zymohexase activity was determined colorimetrically and expressed in terms of optical density of the chromogen as described previously.

membranes contain an enzyme system which catalyses the anaerobic dismutation of HDP at a greater rate than those of the uninfected membranes. It seemed particularly worth while to find out which component of the multi-enzyme system of the membrane is responsible for this increase in glycolytic rate. Although the answer to the question is by no means complete, it is possible to demonstrate a rise of zymohexase activity in centrifuged extracts of infected chorio-allantoic membranes. The increase in zymohexase activity is already present 24 hr. after virus inoculation, at a time when the rate of HDP dismutation is not yet elevated (Table II). The possible significance of these findings will be discussed later.

It was of obvious importance to determine whether or not the virus preparation itself had any enzyme activity. In further experiments, myxoma virus was isolated by differential centrifugation from pooled infected chorio-allantoic membranes. A water-clear suspension of myxoma elementary bodies with an infective titre of 10^{-9} was tested for glycolytic enzyme activities. No enzyme activity could ever be detected, which is in accord with other evidence concerning the lack of any enzyme activity in viruses (Bauer, 1949).

Temporal correlation of morphologic and biochemical changes.

In infection with myxoma virus, an attempt was made to correlate the morphologic changes, both gross and microscopic, with virus titre and metabolic events. The results are presented in Table III. It can be seen that the peaks of lactic acid production, HDP dismutation and zymohexase activity are reached at 96–144 hr., a time when the multiplication of virus is well along in the phase of decline and the titre of virus (10^{-4}) is approximately 1 per cent of its maximum value (10^{-6}).

TABLE III.—*Correlation between Myxoma Virus Titre and Morphological Changes.*

Hours after inoculation.*	Virus titre.	Appearance of chorio-allantoic membranes. Gross appearance.	Microscopic changes.
2	10^{2}	Normal	Normal.
5	10^{2}	„	Hyperplasia of ectoderm ; no inclusions. Immigration of leucocytes into mesoderm. Blood vessels more numerous.
8	10^{1}		Many small bright acidophilic granules present in cytoplasm of ectodermal cells. Increase in size of nuclei in ectodermal cells.
12	10^{2}	„	Very many acidophilic granules in ectodermal cells. Many capillaries plastered against basement membrane of ectoderm. Areas of inflammation throughout mesoderm, specially around blood vessels. Many eosinophilic leucocytes.
24	10^{4}	Membrane oedematous.	Enormous hyperplasia of ectoderm, many mitotic figures. Many ectodermal cells with large acidophilic masses.
36	10^{5}	Innumerable focal lesions present, with oedema of entire membrane	Numerous large acidophilic inclusions in hyperplastic ectoderm. Entire membrane thickened, due to inflammation in mesoderm. Frequent thromboses of small blood vessels. Endoderm also hyperplastic.
48	10^{5}	Lesions similar, more pronounced	Still many mitotic figures in ectodermal cells. Inclusions larger, fewer in number. Hyperplasia in all 3 layers. Infolding of ectoderm. Many eosinophilic leucocytes.
72	10^{5}	Lesions confluent	Inclusions large, infrequent, irregular in shape. Many areas of focal necrosis of ectoderm.
96	10^{4}	Confluent lesions. Gross lesions of maximal intensity at this time	Inclusions and eosinophilic leucocytes much rarer. Whole picture more orderly.
144	10^{4}	Little change. Focal lesions still apparent. Membrane thick, almost leathery	Endodermal hyperplasia almost gone. Ectoderm now looks rather like skin. Inclusions very rare, now large and pale lavender.
192	10^{3}	Membrane still thick	No inclusions found, much sloughing of necrotic debris. Organizing thromboses.

* Embryos of 8 days' incubation used in this experiment.

Biochemical changes in membranes inoculated with viruses other than myxoma.

One of the questions to be answered was whether the reproducible change in the metabolic pattern detectable during virus multiplication is specific for myxoma virus. This problem was approached by comparing the effect of several other viruses on respiration, aerobic lactic acid formation and on the rate of HDP dismutation. Chorio-allantoic membranes were infected with suspensions of fibroma, herpes, vaccinia, Rous sarcoma, influenza and Newcastle viruses; after 2 days' incubation, enzyme assays were done as before. The results are summarized in Table IV. Since the time course of biochemical changes must be

TABLE IV.—*Effect of Several Viruses on Membrane Respiration and Anaerobic HDP Dismutation by Chorio-allantoic Membrane Homogenates.*

Hours after infection.	Virus.	Q_{O_2}.		Q_{CO_2}.	
		Normal.	Infected.	Normal.	Infected.
48	Fibroma	15	13	15	25
„	Herpes simplex	10	8	10	23
„	Vaccinia	10	11	10	20
„	Rous sarcoma	14	12	9·6	20
„	Swine influenza	13	10	9·2	18·2
„	Newcastle	11	12	11	18

expected to vary from one virus to another, the comparison was carried out at a constant time after virus inoculation. As a consequence, the data permit only qualitative comparisons between the different viruses studied. In each case, 48 hr. after infection the O_2 uptake of the whole membranes was unaltered, while HDP fermentation by homogenates was markedly activated.

Recent experiments with a strain of rabies virus adapted to the chorio-allantoic membrane have shown biochemical responses on the part of the membrane identical with those described above.

Our observations yield no biochemical basis for differentiating the action of one virus from that of another. All viruses tested produced increased glycolysis despite marked differences in gross and cytological appearance of the lesions. The very fact that this increase in glycolysis is common to many different viruses may actually provide a useful indication of virus growth, particularly for certain viruses which do not produce grossly detectable lesions on the chorio-allantois.

Biochemical changes in membranes inoculated with Celite and with heat-inactivated virus.

If it is characteristic of the virus-infected chorio-allantoic membrane to exhibit an increase in glycolysis, apparently due to an increase in the rate of HDP dismutation, then this type of response should be absent from membranes showing only " sterile " inflammation. This point was tested by studying the effect of " sterile inflammation " caused by Celite suspensions. The results are summarized in Table V. It is seen that Celite inflammation results in a depression of O_2 uptake and an increase in aerobic lactate production. The Q_{CO_2} values from HDP are not appreciably altered, which suggests that the increased aerobic lactate production is the direct sequence of inhibited tissue respiration.

Although the comparison of these experiments with the ones carried out with active viruses is admittedly difficult, yet the differences observed are at least

TABLE V.—*Effect of Celite on Respiration, Aerobic Lactic Acid Formation and Anaerobic HDP Dismutation by Chorio-allantoic Membranes.*

	Hours after inoculation.	Normal.	Celite 0·5 g. in 10 ml. saline.	Normal.	Celite 0·25 g. in 10 ml. saline.	Normal.	Celite 0·1 g. in 10 ml. saline.
Q_{O_2}	48	17	11	12·6	8·6	8·9	8·3
	96	—	—	—	—	7·0	6·3
	144	—	—	—	—	6·2	6·55
Q Lactic acid	48	16	22	19	22	21·6	19·2
	96	—	—	—	—	11·1	17·7
	144	—	—	—	—	15·9	20·0
Q_{CO_2}	48	20	16	16·1	14·4	11·0	5·0
	96	—	—	—	—	10·3	5·0
	144	—	—	—	—	11·6	15.0

indications that the biochemical response of the chorio-allantoic membrane to actively multiplying viruses is qualitatively different from these " controls."

A second type of " control " experiment was performed in which virus suspensions were placed in sealed ampoules and immersed for 5 min. in a water bath (at 65°, 75° or 100°) to destroy their infectivity. The myxoma suspension used was a water-clear preparation of elementary bodies obtained by differential centrifugation and having a titre of 10^{-9} ; the Rous sarcoma and influenza virus suspensions were lightly centrifuged homogenates of infected chorio-allantoic membranes. Heat-inactivated viruses had no activating effect on glycolysis. Inhibitory effects could be observed on both respiration and aerobic lactic acid production 48 hr. after inoculation with heat-inactivated myxoma and Rous sarcoma viruses. Respiration was not markedly altered by heat-inactivated influenza virus and boiled virus suspensions had no effect on metabolism, as measured by our methods (Table VI).

The decrease in respiration and aerobic lactic acid production by heat-treated virus suspensions is difficult to evaluate at present, since some non-specific breakdown product of virus protein may exert this influence. It should be mentioned,

TABLE VI.—*Effect of Heat-inactivated Viruses on Oxygen Uptake, Lactic Acid Formation and Anaerobic HDP Dismutation of the Chorio-allantoic Membrane.*

	Hours after inoculation	Normal.	Myxoma inactivated at 65°.	Normal.	Myxoma inactivated at 75°.	Normal.	Rous sarcoma inactivated at 75°.	Normal.	Influenza inactivated at 75°
Q_{O_2}	24	—	—	—	—	—	—	10	12
	48	11·0	6	8·9	12·8	13	10	13	14
	96	—	—	7·0	6·5	—	—	—	—
	144	—	—	6·2	6·3	—	—	—	—
Q Lactic acid	24	—	—	—	—	—	—	14	9
	48	13	8	21·6	11·5	17	12	18	10
	96	—	—	18·3	11·1	—	—	—	—
	144	—	—	15·9	19·5	—	—	—	—
Q_{CO_2}	24	—	—	—	—	—	—	16	12
	48	14	10	11·0	8·0	18	11	15	10
	96	—	—	10·3	8·0	—	—	—	—
	144	—	—	11·6	10·1	—	—	—	—

however, that heat-treated saline extracts of chorio-allantoic membranes had no detectable effect at all on these *in vitro* reactions. Similarly, *in vivo* inoculations of chorio-allantoic membranes with such extracts did not alter respiration or aerobic glycolysis (Kun and Smith, 1950).

DISCUSSION.

In order to elucidate the pathogenesis of virus infections, it is necessary to find out how viruses affect the metabolism of the cells which they parasitize. Since, in multicellular animal and plant hosts, the cells themselves function normally only in intact tissues, the study of a particular tissue under conditions of normal growth and of infection would seem to offer a logical approach to the problem. The chorio-allantois is, for several reasons, an almost ideal tissue for such a study.

Unfortunately, virus proliferation within the chorio-allantoic cells themselves is accompanied by profound changes in the tissue as a whole; in-pouring of oedema fluid, migration of extraneous cells into the area, cellular proliferation and necrosis. Could these processes alone be responsible for the increase in glycolysis observed in virus-infected membranes? The following considerations suggest that they could not:

(*a*) Severe inflammation produced by a non-infective agent did not increase the rate of anaerobic HDP dismutation.

(*b*) The increase in glycolysis does not seem to be linked to the type or to the intensity of the cytological response.

Thus these data lend further support to those presented by Bauer (1947, 1948, 1951) for the fact that virus infection can increase the activity of certain enzyme systems, such as dehydrogenases, deaminases, nucleosidases and nucleotidases. Like Bauer (1947), but by a different method, we were also able to show that these metabolic changes are independent of the immigration of leucocytes. Perhaps the most conspicuous observation is the "non-specific" nature of the metabolic response, as determined by our biochemical methods. Although the microscopic changes greatly differ from virus to virus, there is apparently a uniform reaction in the pattern of carbohydrate metabolism.

The lack of effect on overall respiration, however, by no means indicates that the change in the metabolic pattern of infected membranes is limited to the reactions described in this paper. It seems quite likely that a series of enzyme systems competes for the terminal respiratory enzymes and that an alteration of one or more of the metabolic pathways may occur without necessarily affecting the overall O_2 uptake. Indeed, evidence for this point of view is already at hand: recent experiments demonstrate the existence in normal chorio-allantoic membranes of a hexose monophosphate oxidase, in addition to the Emden-Meyerhof-Warburg type of glycolysis and the citric acid cycle (Kun, 1953). Furthermore, respiratory quotient determinations and inhibitor studies indicate that the endogenous respiration of the normal chorio-allantois is largely due to fatty acid oxidation (Kun, unpublished).

It should be borne in mind that the increased breakdown of HDP observed in homogenates may reflect either the actual increase in the amount of certain enzymes, or the activation of inactive enzymes. In either case, the observable result in the test system is an increased rate of HDP breakdown (in homogenates), or an increase in lactic acid formation (by intact membranes). A further source of difficulty in interpreting results is the fact that the test system, by its very

nature, predetermines the direction of any given reaction within a multi-enzyme system; thus the results do not necessarily reflect the actual situation in the intact functioning membrane in its physiological environment.

With these reservations in mind, it seems safe to say that the early appearance of increased zymohexase activity followed by the reversible increase in glycolysis is part of the physiological response to virus synthesis. The increased activity of certain glycolytic enzymes is probably concerned with the synthesis of carbohydrate components (pentoses) of the virus. Cohen (1947) found that the infection of *Escherichia coli* with T2r bacteriophage resulted in a prompt change in the metabolism of the bacterium, consisting of inhibition in growth and a shunt of enzyme reactions in favour of the synthesis of virus components. No change in overall O_2 uptake occurred. Concomitantly a preponderance of anaerobic glucose-6-phosphate utilization was observed.

The increase in HDP dismutation is only one of the mechanisms disturbed during the establishment of a virus infection in the chorio-allantoic membrane. To understand how the infecting virus brings about this increased glycolysis of fructose-1-6-diphosphate, and how the increased glycolysis in turn perhaps provides conditions favourable to virus multiplication, presupposes a clearer concept than we now have of the metabolic pathways of the normal uninfected chorio-allantoic tissue.

SUMMARY.

Metabolic studies were made of chorio-allantoic membranes infected with the following viruses: myxoma, fibroma, Rous sarcoma, swine influenza, Newcastle disease, vaccinia, and herpes simplex. Infection with all viruses tested brought about an increase in anaerobic hexose diphosphate dismutation. Heat-inactivated virus suspensions did not produce this effect. Marked inflammation produced by inoculation of a sterile suspension of Celite was similarly unaccompanied by such a primary effect upon glycolysis.

It is concluded that increased hexose diphosphate dismutation is in some way closely linked with virus multiplication.

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RESEARCHES ON ANTIDIPHTHERIAL SERUM. THE AVIDITY OF PURIFIED SERUM FRACTIONS.

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IN previous papers we have shown that the antitoxin value of an antidiphtherial serum is not always the same when standardised with toxins derived from different sources (Hata and Barksdale, 1951 ; Barksdale and Hata, 1951 ; Hata, 1952). This has been explained by the variability in combining power of an antitoxic serum against different toxins, and by the fact that the value of a toxin varies when tested against different batches of antiserum. These properties were termed avidity and affinity respectively, and their indices defined as follows :

Units-ratio.—The ratio of the antitoxin value of a serum, determined using an arbitrarily selected standard toxin, to the antitoxin value of the same serum found with another toxin. This differs from other indices of avidity in the intimate relation to the antitoxin value.

Affinity-factor.—The ratio of the standard units-ratio of a serum against the standard toxin (taken as 1), to the units-ratio of the same serum determined against another toxin.

For therapeutic purposes an ideal serum would be one with the same avidity for any batch of toxin, having in every case a units-ratio of 1, and such a serum could be expected to neutralise naturally formed toxin in accordance with its labelled value. Our previous work did not suggest that crude antitoxin had these properties, and we were led to test the avidity of fractionated antitoxins to see if they more closely resembled an ideal serum. Barr and Glenny (1931) reported that the serum fraction which contained larger molecules of protein was more avid than that consisting of smaller protein molecules, from comparison of the serum ratios. Kekwick, Macfarlane, Knight and Record (1941) stated that the γ-component of antidiphtherial serum was more avid than antitoxic β-globulin in respect to Kf and serum ratio, and Kekwick and Record (1941) showed that antidiphtherial serum from a horse hyperimmunised for a short period contained larger amounts of γ-globulin. Recently Barr (1949) examined the avidity of antidiphtherial serum against various toxins by the dilution method. It is of interest that her results are similar to ours, although a different technique was used.

The main object of this paper is to record the results of our work on the avidity of fractionated antidiphtherial serum against various toxins derived from different sources.

MATERIALS AND METHODS.

Toxins.

Six batches of antidiphtherial toxin were used, 2 of which were prepared from the Toronto strain of *Corynebacterium diphtheriae* in different media (Mueller and Miller's broth and

Pope's semi-synthetic medium). The remaining four toxins were derived from 2 mitis and 2 gravis strains grown in the same conditions (P.G.T. broth (Hata, 1951)). All the toxins were the same as those used previously and their properties are summarised in Table I. As a test dose, Lr/200 dose was used in each case, and was determined with the same Japanese standard serum prepared in the National Institute of Health, Tokyo.

Antitoxins.

Four antidiphtherial sera were fractionated, of which 3 (H–1–a, 118 and 202) were the same as those studied before. Particulars of these sera are given in Table II. These crude sera were divided into low (30 per cent saturated ammonium sulphate) and high salt (30–50 per cent saturated ammonium sulphate) fractions, and each fraction was dialysed against running tap-water until free from sulphate, adjusted to half the initial volume, and the pH adjusted to 7·2. Sodium chloride, 0·8 per cent, and sodium ethyl-mercurithio-salicylate (Merzonin, " Takeda "), 0·02 per cent, were added, and the solution sterilised by passage through a Seitz filter. In the Figure are displayed the electrophoretic patterns of the 4 antisera and their fractions.

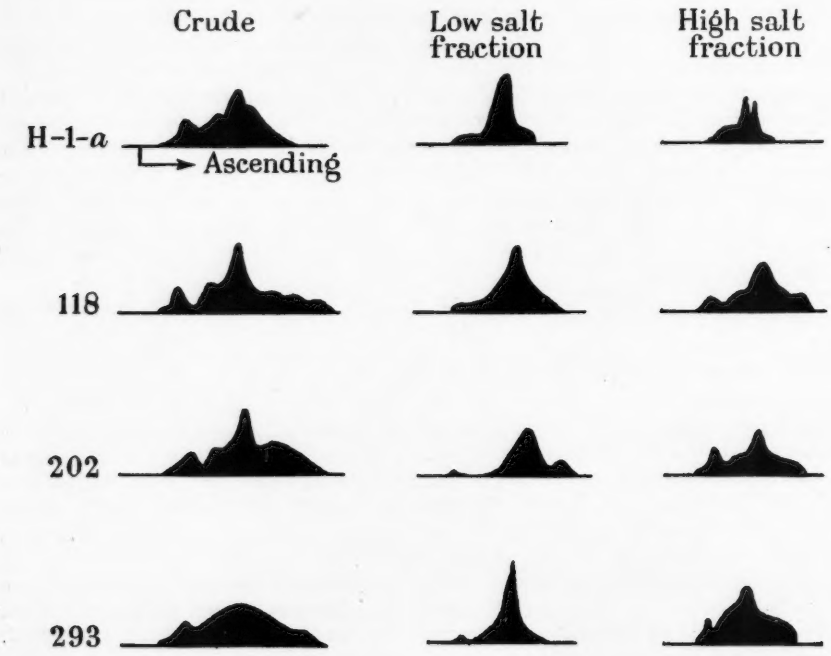

FIGURE.—Electrophoretic patterns of antidiphtherial sera and fractions thereof.

The technique was the same as that used in previous work. As a control, the standard serum (6), which had been employed for the determination of the test dose of each toxin, was titrated with the same test dose at the close of the experiment. The technical error in titration is within ± 3 per cent: guinea-pigs were the test animals. The Toronto toxin T–P–13 was selected as a standard toxin, with which the standard antitoxin value of each serum fraction was determined. The units-ratio and the affinity factor were then calculated.

TABLE I.—*Diphtherial Toxins.*

Toxin.	Strain.	Type.	Medium.	Date prepared.	Lr/200 dose* in ml.
T–H–2	Toronto	Mitis	Mueller and Miller's	18.ii.49	0·0001
T–P–13	,,	,,	Pope's	10.ii.50	0·0003
TG–11	TG–11	Gravis	P.G.T.	30.iii.50	0·0070
KG–1	KG–1	,,	,,	18.ii.50	0·0100
TM–8	TM–8	Mitis	,,	11.iv.50	0·0060
M–H–2	53–A–4	,,	,,	,,	0·0120

* The figures indicate the test-doses used in these experiments (Sept.–Oct., 1951).

TABLE II.—*Crude Antidiphtherial Sera.*

Serum.	Horse.	Antigen.	Hyper-immunization begun.	Date bled.	Antitoxin *in vivo* μ/ml.	Value* *in vitro* μ/ml.	N mg./ml.†
H–1–a	H–1	Gravis toxoid	20.i.49	5.vii.49	15	16	13·54
118	43	Toronto toxin	10.x.49	11.i.50	110	700	14·46
202	58	,, ,,	25.xi.49	26.i.50	390	750	15·52
293	Pool	,, ,,	—	—	1100	1200	12·93

* The antitoxin value was standardised with the "standard toxin" (T–P–13) just before the experiment (Sept. 7th, 1951).

† Total nitrogen content of the serum was determined using a micro-Kjeldahl method.

TABLE III.—*Difference in Antitoxin Values of the same Antidiphtherial Serum Fraction Standardised with Different Test Toxins.*

Serum.	Nature.	Standard† antitoxin value. A.U./ml.	Fraction.	Test toxin. T–H–2. A.U./ml.	T–P–13. A.U./ml.	TG–11. A.U./ml.	KG–1. A.U./ml.	TM–8. A.U./ml.	M–H–2. A.U./ml.	Standard† antitoxin value. A.U./ml.	Yield.‡ In per cent.
H–1–a	Antigravis	15	L*	6·5	7·0	7·0	5·5	5·5	6·0	7·0	55·0
			H	8·5	9·5	10·5	10·0	10·0	10·0	9·5	
118	Anti-Toronto	110	L	25	20	15	15	10	15	20	45·5
			H	100	80	80	80	60	60	80	
202	,,	390	L	150	175	100	125	125	150	175	25·6
			H	20	25	15	15	20	20	25	
293	,,	1100	L	600	600	600	500	500	500	600	68·2
			H	800	900	1000	1000	1000	1000	900	
6	Standard serum	10	C	10	10	10	10	10	10	10	

* L = Low salt fraction. H = High salt fraction. C = Crude serum.
† Standard antitoxin value = the value that was decided with the standard toxin (T–P–13).
‡ The yield was calculated from "standard antitoxin value."
The experimental error in titration of antitoxin value is within ± 3 per cent.

RESULTS.

The antitoxin values of the purified fractions and the standard serum, 6, standardised with 6 different toxins are recorded in Table III, the figures for the initial crude antiserum being those obtained in experiments carried out 7 months earlier. As toxin T–P–13 was selected as the standard toxin, the figure in this column represents the standard antitoxin value. The antitoxin value of the standard serum, 6, is constant even if any of the test toxins, of which test doses have been decided with the same standard serum, are employed, indicating that there has been no error in determining these test doses and that there have

been no changes of the test doses throughout the experiment. Values for the units-ratio and affinity factor are shown in Table IV.

As can be seen from the tables, the standard antitoxin value of the low salt (L) fraction of serum H-1-a is 7 A.U./ml. and its units-ratio varies between 1·0 and 0·79 in these experiments. The values for the high salt (H) fraction of the same serum are 9·5 A.U./ml. and 1·11–0·89. In the case of serum 118, L fraction is 20 A.U./ml., 1·25–0·5; H fraction is 80 A.U./ml., 1·25–0·75. In serum 202, the L fraction results are 175 A.U./ml., and 1·0–0·57, and for the H fraction 25 A.U./ml., and 1·0–0·6. In serum 293, the L fraction is 600 A.U./ml., and 1·0–0·83, and the H fraction is 900 A.U./ml., 1·1–0·89.

These results suggest that antitoxic serum fractions purified by the salting-out method may not always have the same avidity for different batches of toxin, and that it has not been possible to prepare an ideal serum from the crude antiserum. Comparing the avidities of the L and H fractions of each serum, all the H fractions were more avid than the L fractions with exception of serum 202. This serum showed marked differences in avidity when tested against the different toxins, and it is of interest that the L and H fractions prepared from it show similar differences in units-ratio, although to a lesser degree. It was also abnormal in that the L fraction contained considerably more protein than the H fraction (Table VI). Turning to the affinities of the toxins against each fraction it will be seen that the two toxins prepared from the Toronto strain of *C. diphtheriae* came first and second in affinity order, and that all the toxins combined more easily with the H fractions except toxin T–H–2 (Tables IV and V).

During this work intradermal reactions showing double and ring flushes were often seen. They occurred 9 times with toxin TM–8, 7 times with KG–1 and M–H–2, 6 times with T–H–2 and TG–11, and only 3 times with T–P–13.

TABLE IV.—*Avidity of Serum Fraction and Affinity of Toxin.*

Serum.	Fraction.	Toxin. T–H–2.	T–P–13.	TG–11.	KG–1.	TM–8.	M–H–2.	Units-ratio. Mean.	Order.
	Units-ratio→								
H–1–a	L*	0·93	1·00	1·00	0·79	0·79	0·86	0·90	5
	H	0·89	1·00	1·11	1·65	1·05	1·05	1·03	2
118	L	1·25	1·00	0·75	0·75	0·50	0·75	0·83	6
	H	1·25	1·00	1·00	1·00	0·75	0·75	0·96	3
202	L	0·86	1·00	0·57	0·71	0·71	0·86	0·79	7
	H	0·80	1·00	0·60	0·60	0·80	0·80	0·77	8
293	L	1·00	1·00	1·00	0·83	0·83	0·83	0·92	4
	H	0·89	1·00	1·10	1·10	1·10	1·10	1·05	1
←Affinity factor	Mean	0·99	1·00	0·89	0·87	0·82	0·88		
Affinity factor	Order	2	1	3	5	6	4		

* L = Low salt fraction. H = High salt fraction.

TABLE V.—*Comparison of Avidity of Both Globulin Fractions against Each Toxin.*

Test toxin:	T–H–2.		T–P–13.		TG–11.		KG–1.		TM–8.		M–H–2.	
Serum. Serum fraction:	L.*	H.	L.	H.	L.	H.	L.	H.	L.	H.	L.	H.
H–1–a	0·93	0·89	1·00	1·00	1·00	1·11	0·79	1·05	0·79	1·05	0·86	1·05
118	1·25	1·25	1·00	1·00	0·75	1·00	0·75	1·00	0·50	0·75	0·75	0·75
202	0·86	0·80	1·00	1·00	0·57	0·60	0·71	0·60	0·71	0·80	0·86	0·80
293	1·00	0·89	1·00	1·00	1·00	1·11	0·83	1·10	0·83	1·10	0·83	1·10
Units-ratio. Mean value	1·01	0·96	1·00	1·00	0·83	0·95	0·77	0·97	0·71	0·93	0·83	0·93

* L = Low salt fraction. H = High salt fraction.

This frequency is almost in accordance with the affinity order of these toxins (Table IV).

The serum fractions were salted out several times to increase the purity of the protein fraction, and as estimated electrophoretically contained little or no albumen, the L fraction consisting mainly of γ component and the H fraction of α, β, and γ components. The poor antitoxin value of these fractions may well be related to the purification process and to the final Seitz filtration.

TABLE VI.—*Purity (A.U. per g. Protein) of the Antitoxic L and H Globulin Fractions and Their Original Crude Sera.*

Serum.	Fraction.	Test toxin. T–H–2.	T–P–13.	TG–11.	KG–1.	TM–8.	M–H–2.	Protein content (per cent).	Ratio H/L.
H–1–a	Crude*	130	177	177	154	142	130	8·46	
,,	L†	253	272	272	214	214	233	2·57	2·08
,,	H	159	178	197	187	187	187	5·34	
118	Crude	1,438	1,217	995	774	1,106	1,106	9·04	
,,	L	796	637	478	478	318	478	3·14	2·64
,,	H	1,208	966	966	966	725	725	8·28	
202	Crude	1,856	4,021	1,237	1,959	1,649	1,753	9·70	
,,	L	1,678	1,953	1,119	1,398	1,398	1,678	8·94	0·25
,,	H	840	1,092	630	630	840	840	2·38	
293	Crude	—	13,614	—	—	—	—	8·08	
,,	L	14,670	14,670	14,670	12,225	12,225	12,225	4·09	1·33
,,	H	14,760	16,605	18,450	18,450	18,450	18,450	5·42	

* The antitoxin value employed for the calculation of the purity was quoted from the previous report (Hata, 1952).
† L = Low salt fraction. H = High salt fraction.

In Table VI may be seen the protein content of the L and H fractions and of the original crude antiserum, as well as the purity (antitoxin value per g. protein) of these substances. It shows that for serum 202 the protein content of the L fractions was greater than that of the H fractions. It will be noted that the antitoxic purity of all fractions of remarkably avid serum (118 and 202) was inferior to that of the corresponding crude serum. In respect to antitoxin values the L fraction of serum 202 contained much more than its H fraction ; in serum 293 the latter contained relatively more than the former, and in serum 118 considerably more. Thus the distribution of L and H globulins in the crude antiserum seems to be roughly in accordance with that of antitoxin in both globulins, although this was not the case with H–1–a whose antitoxin value was remarkably low against every toxin. The L fraction of this serum contained 1·59 times more antitoxin than the H fraction, while the crude serum contained 2·08 times more H globulin than L globulin. It may be that the evidence is insufficient to say that the antitoxin in the low-titre antiserum is mainly demonstrable in the L fraction.

DISCUSSION.

In our previous studies we have shown that the combining power of a crude antidiphtherial serum was not always the same against different diphtherial toxins, and that the same was true in the case of a toxin against different batches of antiserum. We are grateful to Dr. B. Cinader and Mr. B. Weitz, of the Lister Institute of Preventive Medicine, for a personal communication to the effect that similar observations have been made in the tetanus toxin-antitoxin system. The results reported by Barr (1949) that the dilution ratio of a serum is different against various test toxins means that the serum has different units-ratios.

Before discussing the variety of antitoxin protein molecules, it was decided to see if the difference of avidity or affinity would be affected by non-antitoxic or non-toxic proteins or other substances contained in crude samples. As a first step we have worked with low and high salt antitoxic fractions. These have been found to vary in their reaction with different batches of toxin in the same way as crude antiserum, and this means that antitoxic serum fractions prepared by a salting out process may not always neutralise different batches of toxin to their standard antitoxin values. We cannot from this conclude that commercial purified antitoxin is superior to crude antiserum in respect to avidity. The avidity of each antitoxic fraction or the mean of the avidities of both fractions of the same serum were not always proportional to the avidity of the corresponding crude serum. This is probably due to the considerable loss of antitoxin during purification.

Barr and Glenny (1931) and Kekwick *et al.* (1941) have stated that the euglobulin (γ component) is more avid : more recently Levine (1952) claims that the avidity of γ globulin is nearly equal to that of the β_2 fraction. These authors have evaluated avidity using time of flocculation and serum ratio. In our results the low salt globulin fraction was in general less avid than the high salt fraction. These discrepancies may be due to differences in methods of assay, and it is also doubtful if it is justifiable to compare the avidities of low and high salt globulin fractions.

The Toronto toxins had the highest mean value of affinity factor, but it is doubtful if the Toronto toxin has stronger affinity against the serum obtained by hyperimmunising with the same antigen, as the number of antitoxic fractions tested is very limited. This point we discussed in previous publications.

Barr (1951) stated that ring intradermal reactions are rare in the titration of non-avid samples of antitoxin, and we have found the appearance of double and ring flushes in titrations with crude antiserum to be closely correlated with the affinity factor. We would prefer to discuss this problem after the accumulation of much more data.

In this paper work on the avidity of antitoxic serum fractions has been described. It is proposed to repeat these experiments using more highly purified antitoxin, prepared by digestion and alcohol fractionisation, and experiments with digested material are already in progress.

SUMMARY.

Four batches of antidiphtherial sera were divided into low and high salt fractions, and each fraction was purified by repeated salting out. The avidity of the purified fraction was studied using six different toxins, and it was clear that the antitoxin value of the fraction was not always the same when assayed with different toxins.

Under the limited experimental conditions, avidity (average units-ratio) of the high salt fraction was generally greater than that of the low salt fraction, but this was not always so. While it may be possible to compare the avidity of individual fractions, any discussion on the superiority of one fraction over another is liable to be inconclusive.

Salting out with ammonium sulphate is not a satisfactory way of preparing an ideal serum, and this raises the question whether crude serum should not be

used together with refined serum in the treatment of malignant forms of the disease.

Toxins causing double or ring intradermal reactions are likely to have poor affinity.

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FLUORESCENCE AND ABSORPTION SPECTRAL DATA FOR PTERIN ISOLATED FROM THE TUBERCLE BACILLUS BY CHROMATOGRAPHIC ANALYSIS.

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THE pterins are a group of pigments having a pteridine ring system (fused pyrimidine and pyrazine rings) which were first isolated from the wings of butterflies by Hopkins (1895, 1941–42). Wieland and Schöpf (1925), Wieland and Purrmann (1939), Schöpf and Becker (1933, 1936), Becker and Schöpf (1936), Schöpf and Kottler (1939), and others also isolated the pigments from the wings of butterflies and from the integument of other insects. The pterins present exceptional difficulties in their separation and purification because they usually occur in mixtures, have no melting points, and cannot be transformed into derivatives with definite melting points.

One of this group of pigments, xanthopterin, which is characteristic of the brimstone butterfly (*Gonepteryx rhamni*) has become of special significance in recent years due to reports of its presence in human urine, its haemopoietic activity and its use in nutrition, and other fields of biology and medicine. Recently, it has been reported that xanthopterin is of " intrinsic importance in regulating normal cell division and may play a part in the continuous multiplication of tumour cells " (Editorial, 1952 ; Report, 1947). Xanthopterin has also produced a " striking inhibition of psittacosis virus growth at 0·1 mg./ml." (Morgan, 1952).

Xanthopterin was synthesized for the first time by Purrmann (1940, 1941). This work was later of great value in the elucidation of the structure and the synthesis of pteroylglutamic acid (folic acid, *L. casei* factor from liver) (Angier *et al.*, 1945, 1946). The nuclear structures of xanthopterin and pteroylglutamic acid are identical.

Evidence already has been presented (Crowe and Walker, 1949) that an erythropterin-like pigment is derived from the human tubercle bacillus. Combined chromatographic and fluorescence analyses of extracts of dried bacilli also revealed the presence of yellow fluorescing zones in the chromatograms, indicating the presence of pigments other than riboflavin which is known to be present in extracts of tubercle bacilli (Street and Reeves, 1940 ; Boissevain, Drea and Schultz, 1938 ; Rohner and Roulet, 1939).

Evidence is here presented that a pterin, having all the chief characteristics of xanthopterin, is synthesized by the virulent human tubercle bacillus grown in synthetic medium. This has not, to our knowledge, been reported before.

MATERIAL AND METHODS.

Strains and cultural methods.

Myco. tuberculosis var. *hominis* 48189 isolated from gastric contents collected in July, 1947, was the main source of material, but strains 13 and 48214, obtained from Dr. R. J.

Dubos, Rockefeller Institute, labelled H37Rv, were also used for supplementary and corroborative data.

Myco. tuberculosis 48189 was cultivated for 6 weeks at 37° in Long's synthetic medium (asparagine, 5 g.; ammonium citrate, 5 g.; KH_2PO_4, 3 g.; Na_2CO_3 anhydrous, 5 g.; NaCl, 2g.; $MgSO_4$, 1g.; ferric ammonium citrate, 0·05g.; glycerol, 50g.; distilled water to 1 l. vol.); strain 13, for 3 weeks at 37·5° in beef infusion broth with glycerol, medium *F*8 (Wadsworth, 1947); and strain H37Rv, for 16 days at 37° in Dubos' medium (Dubos and Middlebrook, 1947), very slightly modified (basal medium No. 7).

Methods of extraction.

Various solvents are used for the extraction of dried bacilli as steps in the preparation of antigens used in the complement fixation test for tuberculosis (Wadsworth, 1947). An acetone extract, approximately 3800 ml. of about 150 g. dry wt. of heat-killed *Myco. tuberculosis* 48189, was available for our general studies of pigments synthesized by this organism and was the chief source of the material for which physical data are presented.

Supplementary data were obtained for the following further extracts of dried bacilli: strain 48189 (28·0 g.), acetone extract, strain 13 (28·0 g.), methanol extract, strain H37Rv (36·6 g.), acetone extract.

Chromatographic methods.

Exploratory experiments to determine suitable solvents and elutriants were performed with small samples of the extracts using a micromethod of chromatographic analysis (Crowe, 1941) with aluminium oxide, standardized according to Brockmann and Schodder (1941) by Merck and hereafter called alumina. This micromethod was also useful in its application to paper chromatography: drops of various solutions of pigment were placed at the edge of filter and blotting papers and solvents were selected which were suitable for developing chromatograms on the papers.

The columns of alumina used later to obtain fractions of the extracts were usually small, about 5–10 cm. in height and 0·8 cm. in diameter. Only small volumes of solutions, 3–10 ml., were used because better separation of zones and recovery of material were so attained. Fractions were sometimes passed through several columns successively until chromatographic homogeneity was attained. Hence the yield of material was small, and it was necessary to obtain data for the various physical characteristics of the pigment from different samples.

Spectroscopic methods.

Fluorescence and absorption spectral data were obtained for some of the purified material. The fluorescence spectrograms were recorded on Kodak panchromatic and spectroscopic Type 1 L plates, red-sensitive, with a Hilger medium quartz Spectrograph E 316. The slit width was 0·15 mm. Radiations from a Hanovia analytic quartz lamp were used to excite the fluorescence in samples contained in glass cells which were placed approximately 1 cm. before the slit of the spectrograph and 10 cm. from the lamp. Helium was used for the reference spectrum. The spectrograms were evaluated by tracings made with a Zeiss recording microphotometer. Measurements of the tracings for wave-length determinations of band maxima were made with a Hilger measuring micrometer. Spectrophotometric data in the ultraviolet region were recorded on Kodak spectroscopic plates Type 1 L, Kodak panchromatic, and Kodak 33 plates with the quartz spectrograph and Spekker photometer. The light source was a tungsten steel condensed spark. The slit width was 0·04 mm.

Absorption spectral data in the range λ 8–15μ were obtained with a Perkin-Elmer recording infra-red spectrophotometer.

RESULTS.

Preparation of fractions.

The amber-coloured acetone extract, 3800 ml. of 150 g. of heat-killed *Myco. tuberculosis* 48189, was reduced to about 950 ml. by evaporation at room temperature. The extract became yellow as globules of reddish-brown viscous material formed and settled to the bottom. This substance was separated by decanting

the yellow solution, which fluoresced with a yellow light, and was stored in a petroleum-ether 1 : 1 mixture for future study.

Exploratory experiments with the yellow solution on columns of alumina revealed several zones of blue, green, and red fluorescing pigments in addition to zones of yellow fluorescing pigments. None of the media showed such zone formations when passed through similar columns. The separation of these pigments and the isolation and purification of the yellow fluorescing material were therefore attempted.

The entire yellow solution was shaken with a petroleum-ether 1 : 1 mixture without the addition of water, which was found by experiments with test samples to be unnecessary. The petroleum-ether phase, which fluoresced with a red light, was separated from the yellow hypophase, designated as fraction A, which fluoresced with a greenish-yellow light. Glacial acetic acid was added to fraction A to give a 10 per cent concentration of acetic acid ; this was extracted first with petroleum ether 1 : 1 mixture, in order to remove any residual red fluorescing pigment, and then with chloroform. In each case the extraction removed a blue fluorescing material. Fraction A, thus extracted and filtered twice through Büchner funnels and passed through two chromatographs of alumina, was treated with pellets of sodium hydroxide, and this solution was designated fraction B.

Preparation of crystalline salts.

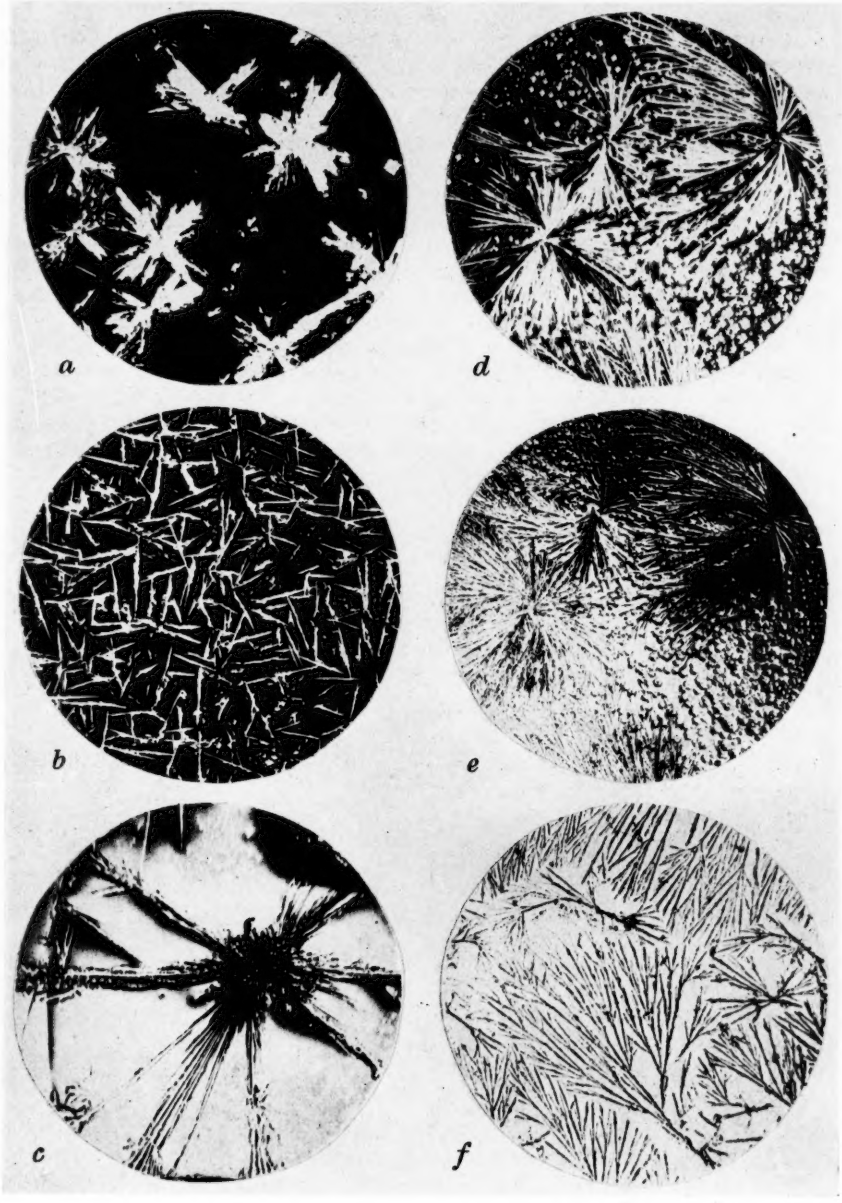
Sodium salts.—While the exploratory experiments were in progress, a small amount of flocculent material, brown-yellow in colour, gradually formed in fraction B and was separated from the solution and used for studies of crystalline forms. When this flocculent material was dried and dissolved in hot 0·1 N NaOH, crystals appeared as the solution slowly cooled. They were brilliant stellar-shaped clusters of needles when, in the moist state or floating in the mother liquor, they were observed in the microscope with crossed polaroid in the light beam (Fig. 1*a*). The solutions of the flocculent material sometimes yielded only the amorphous forms of the sodium salt which, when thoroughly dried, were entirely blotted out when viewed in the microscope with crossed polaroid. Stellar-shaped aggregates of crystals, however, were formed usually by the concentration of a fresh 0·1 N NaOH solution of the amorphous material. Spindle-shaped crystals (Fig. 1*b*) also were formed, in a similar manner, from more dilute solutions of NaOH.

A sample of fraction B was further purified by pouring the hot solution, treated with a small amount of animal charcoal, into 0·1 N methanolic HCl. The stellar-shaped bundles of crystals of the sodium salt (Fig. 1*c*) were obtained from the mother liquor after it was separated from the precipitated solids.

Barium salts.—The barium salt was first obtained by dissolving residues of samples (10–25 ml.) of fraction B in 2 N NH_4OH, which was then filtered through a Büchner funnel and poured into boiling 0·2 N BaOH. The supernatant was separated from the ppt. by centrifugation or filtration and the ppt. was discarded since spectroscopic examination showed no selective absorption by it. The barium salt obtained from the supernatant in different experiments assumed

EXPLANATION OF PLATE.

FIG. 1.—Photomicrographs (× 45) of the sodium salts (*a*, *b*, *c*) and the barium salts (*d*, *e*, *f*) of the pterin from the human tubercle bacillus (*a*, *b*, *d*, polarized light).



Crowe and Walker.

various forms which were similar to those reported for xanthopterin (Schöpf and Becker, 1933, 1936; Schöpf and Kottler, 1939). They were amorphous, knob-like aggregates, light brown jelly-like material, fine long needles, stellar-shaped aggregates of crystals, and sheaf-like bundles of crystals sometimes interspersed with the barium salt in an amorphous form (Fig. 1*d*, *e*). Some of the more concentrated solutions of the barium salt of the pterin from fraction B, dissolved in 2 N HCl and treated with sodium acetate, frequently yielded only the amorphous form of the barium salt. The more dilute solutions, similarly treated, formed spindle-shaped crystals as in dilute solutions of the sodium salt (Fig. 1*b*) which closely resemble those reported by Schöpf and Becker from β-xanthopterin-barium and designated by them as xanthopterin hydrates *a* and *b*. Moreover, the sheaf-like aggregates of the barium salt, when thoroughly dry (Fig. 1*f*), were optically inactive when viewed in the microscope with crossed polaroid as was reported by the German workers for their β-xanthopterin-barium crystals.

The barium salt was finally prepared in an atmosphere of N_2. The residue of 100 ml. of fraction B was dissolved in 115 ml. of 2 N NH_4OH which was mixed with 300 ml. of boiling 0·2 N BaOH by filtering the solutions successively into a flask containing N_2 so that the entire reaction took place with the exclusion of atmospheric CO_2. The supernatant was separated from the ppt. which was discarded. When the supernatant was concentrated, it yielded sheaf-like aggregates of crystals, characteristic of β-xanthopterin-barium.

Miscellaneous observations.

Other similarities were observed between xanthopterin and the yellow pterin from the tubercle bacillus. When a few crystals of the barium salt were added to a mixture of 6 ml. of acetic acid and 1·2 ml. of 30 per cent H_2O_2, a rapid transformation of the yellow pigment into a crystalline colourless substance took place. This substance, in water suspension, did not have the greenish-blue fluorescence of the original material; but, when heated, the colour and fluorescence returned. Finally, on evaporation of the suspension, the crystals of the barium salt, characteristic of β-xanthopterin-barium formed and thus indicated a re-conversion of the colourless substances to the yellow pterin.

The findings in these experiments agree with those for β-xanthopterin-barium, similarly treated, which were reported by Schöpf and Kottler (1939) and by Wieland and Purrmann (1939). The German workers have pointed out, in respect to this reaction, a similarity between xanthopterin and toxoflavin (van Veen and Baars, 1938), the yellow toxic principle of bongkrek. Wieland and Purrmann also cite in further support of this similarity the close agreement in the wavelength positions of the absorption band maxima for xanthopterin at λ 3950 Å and 3750 Å reported by Schöpf and Becker (1936), and those for toxoflavin reported by Stern (1935).

The solubility of the pterin from the tubercle bacillus in glacial acetic acid, 0·1 N H_2SO_4, 0·1 N HCl, 0·1 N NaOH, NH_4OH, ethylene glycol, and hot water resembles that of xanthopterin. Solutions in various solvents and at different pH values varied in colour from citron-yellow to orange-yellow and the solid residues from pale yellow to deep yellow. Treatment of the pigment with ammoniacal $AgNO_3$ solution gave a brown ppt. that darkened on standing. This result was the same as for xanthopterin when similarly treated.

Spectroscopic data.

Fluorescence spectra.—Microphotometric tracings (Fig. 2) were made of fluorescence spectrograms for samples of the pterin from the tubercle bacillus and for synthetic xanthopterin.

A sample (10 ml.) of fraction B was evaporated by heating. While still moist, the residue fluoresced with a yellow light which was more intense than that of the sample solution. The tracing (Fig. 2*b*) shows two band max. at λ 4459 and

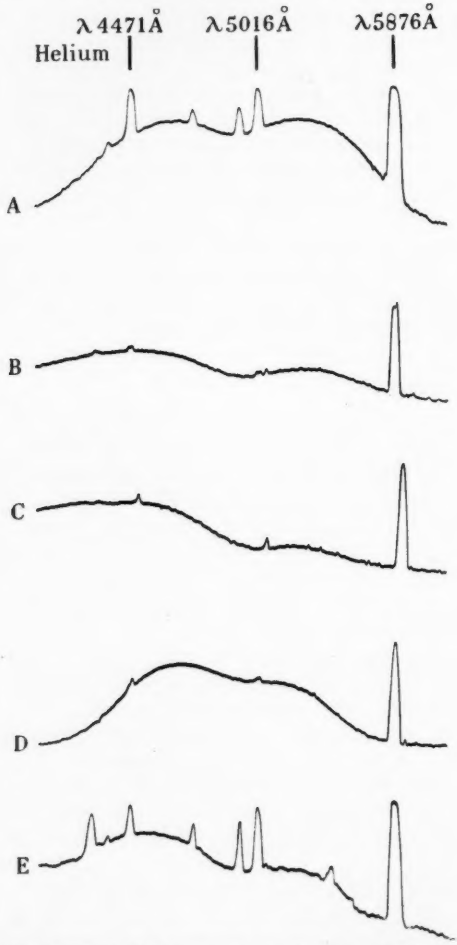


FIG. 2.—Microphotometric tracings of fluorescence spectrograms for the pterin derived from the human tubercle bacillus and for synthetic xanthopterin, showing fluorescence maxima between λ 4471Å and λ 5876Å (*a*) Pterin in aqueous solution (strain 13). (*b*) Moist residue from fraction B (strain 48189). (*c*) Barium salt of pterin in 2 N acetic acid (strain 48189). (*d*) Pterin in 0·1 N NaOH (strain 48189). (*e*) Synthetic xanthopterin (in glacial acetic acid); prepared by Dr. J. R. Totter.

5537 Å. The tracing (Fig. 2*c*) of the spectrogram for the barium salt of the pterin from fraction B in 2 N acetic acid shows two band max. at λ 4312 and 5124 Å. Additional fluorescence spectral data were also obtained as corroborative evidence for samples of the pterin isolated from other tubercle bacilli, namely, from another acetone extract of the same strain, 48189 ; from a methanolic extract of strain 13 ; and from an acetone extract of strain H37Rv.

The acetone solution of the residue, previously extracted with chloroform, of a fraction from another lot of strain 48189, prepared in the same manner as fraction B, was passed through two columns of alumina and the chromatograms were developed with acetone and water successively. The green-yellow fluorescence of the aqueous solution recovered from the second chromatogram was recorded. The tracing indicated band max. at λ 4634 and 5081 Å. The residue of another sample of this solution was dissolved in 0·1 N NaOH and the fluorescence was recorded. The tracing of the spectrogram shows band max. at λ 4657 and 5091 Å (Fig. 2*d*). A methanolic extract was made from the dried bacilli of strain 13 that had been extracted previously with ether and chloroform. The extract was concentrated and passed through two columns of alumina which were developed with methanol and distilled water successively. The aqueous solution obtained from the second column was citron-yellow and fluoresced with a yellow-green light which was recorded on a spectrogram. The tracing (Fig. 2*a*) indicates band max. at λ 4659 and 5265 Å.

An acetone extract of strain H37Rv (36·6 g.) was treated as previously described for other acetone extracts that yielded fraction B. Chromatographic analysis of this material yielded a yellow fluorescing zone on the column which was elutriated with distilled water. The yellow fluorescence of this aqueous solution was recorded. The tracing shows two band maxima which are in about the same positions as the two main bands shown in tracings for the other acetone extracts. When the solution used for the spectrogram was evaporated, sheaf-like bundles of crystals characteristic of xanthopterin formed on the wall of the vessel. Moreover, a tracing of a fluorescence spectrogram for the yellow pterin, while still adsorbed on the column, showed two emission bands which agree closely in the positions of their maxima with those of the yellow fluorescing residue reported for strain 48189.

The microphotometric tracings of all the fluorescence spectrograms made for the pigment derived from tubercle bacilli of different strains and grown in various media show fluorescence band maxima that fall within the range reported for the fluorescence of xanthopterin in acid, neutral, and alkaline solutions (Jacobson and Simpson, 1946). They also agree closely with the band maxima shown in the tracing (Fig. 2*e*) for synthetic xanthopterin prepared by Totter (1944) who kindly furnished us with a sample.

The fluorescence of the barium salt of the pigment dissolved in 0·1 N H_2SO_4 was not quenched when the solution was heated on a water bath for 15 min. The solution thus treated was evaporated in a beaker. The residue showed a brilliant greenish-yellow fluorescence. Sheaf-like aggregates of crystals were formed on the wall of the beaker. The fluorescence was not quenched by ultraviolet irradiation or by exposure to daylight. All of these results are in accord with those reported for xanthopterin (Schöpf and Becker, 1933, 1936).

Absorption spectra.—Absorption spectral curves in the ultraviolet region and in the infra-red range λ8 to 15μ were made for samples of the pterin for which

fluorescence data had been obtained. Some of the flocculent material that precipitated from fraction B was dried and dissolved in glacial acetic acid. The absorption curve for this solution showed a band max. at λ 2587 Å and an inflection in the region λ 3000–4000 Å. A sample of the solution was further purified by passage through two columns. The absorption curve (Fig. 3) for the resulting greenish-blue fluorescing solution showed a band maximum at λ 2595 Å and an inflection in the region λ 3000–4000 Å. The wavelength positions for this chromatographically purified sample are identical with those shown in the curve

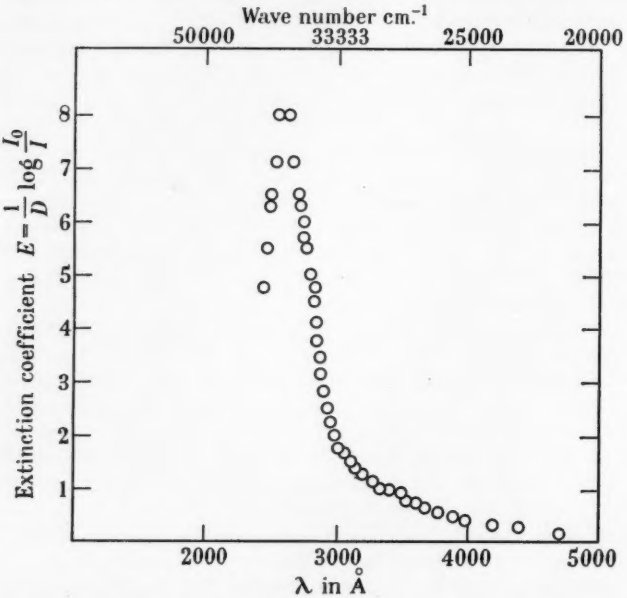


FIG 3.—Ultraviolet absorption spectrum curve. Pterin (in glacial acetic acid) isolated from the human tubercle bacillus.

for a 2 N acetic acid solution of the barium salt of the pigment prepared in an atmosphere of N_2. The aqueous solution, also purified by chromatography and for which fluorescence data have been given (Fig. 2*a*), yielded an absorption curve having a band maximum at λ 2562 Å and two inflections from λ 3000–3400 Å and λ 3700–4050 Å.

The infra-red absorption spectra in the range $\lambda 8$–15μ was recorded (Fig. 4) for the barium salt of synthetic xanthopterin (curve A) and for the barium salt of the pterin isolated from the tubercle bacillus (curve B). There is close agreement between the positions of the band maxima for the two curves.

Chromatographic behaviour.

The sequence and appearance on columns of alumina of mixtures of several pterins—erythropterin, chrysopterin, xanthopterin and leucopterin—have been

reported (Schöpf and Becker, 1933, 1936 ; Becker and Schöpf, 1936). The same sequence of zones has been observed for pigment derived from extracts of the tubercle bacilli strains 48189, 13 and H37Rv used in this report and in previous work (Crowe and Walker, 1949). These observations also have been made in our studies of pigments derived from the diphtheria bacillus (Wadsworth and Crowe, 1943 ; Crowe and Walker, 1944).

Becker and Schöpf (1936) developed a very definite and characteristic adsorption test for the detection of traces of xanthopterin in insects. By the application of a very small quantity of xanthopterin (0·5–1·0 μg.) in not too great a volume of solution (1·5–2·0 ml.) of 0·01 per cent methanolic HCl to a capillary column of alumina (5·0 × 0·1 cm.), a small sharp yellow-green fluorescing zone was adsorbed

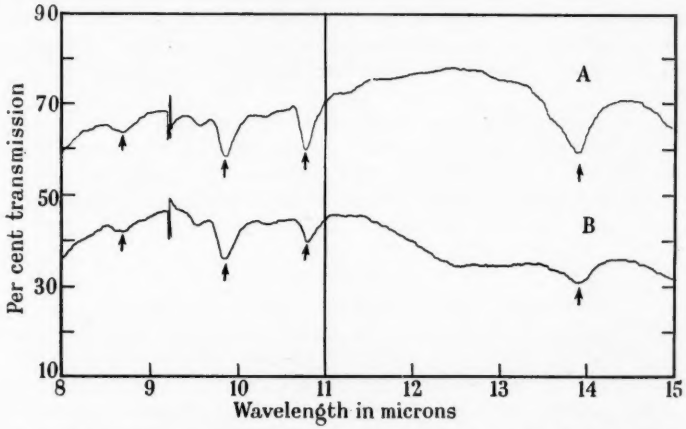


FIG. 4.—Infra-red spectra. A. Barium salt, xanthopterin, synthetic. B. Barium salt, pterin from tubercle bacillus.

near the top of the column. This zone, invisible in daylight, was observed by its fluorescence when the same amount of pigment in dilute aqueous HCl was applied to similar columns. Application of larger amounts than 1μg. of xanthopterin in aqueous HCl, however, caused the pigment to spread over the entire column which fluoresced with a yellow-green light. The adsorptive capacity of alumina for xanthopterin in dilute solution of methanolic HCl is greater than in an aqueous solution. This adsorption test, when applied under similar conditions to the yellow pterin from the tubercle bacillus, yielded the same results as reported by Becker and Schöpf.

DISCUSSION.

All the absorption curves for the yellow pterin from the tubercle bacillus, isolated and purified in various ways, show selective absorption within the limits of those reported by other workers (Jacobson and Simpson, 1946) for xanthopterin, namely, λ 2550–2750 Å and λ 3750–3800 Å. However, although the selective absorption in the curve of Fig. 3 is indicated by a definite band in the region where the main band of xanthopterin occurs, the absorption in the range λ 3000–4000

Å is indicated only as an inflection and the murexide test is negative. Goodwin and Srisukh (1951) also have noted a similar result in their absorption curve for 0·1 N NaOH and glacial acetic acid solutions of a pterin extracted from the eyes of locusts and have also reported a negative murexide test for their pterin.

In our earlier studies of a xanthopterin derived from diphtheria toxin (Crowe and Walker, 1944) a definite band maximum at λ 3785 Å was observed for the pigment in 0·067 M borate buffer, pH 9·2, in contrast with only an inflection in this region recorded for the pigment derived from the tubercle bacillus likewise dissolved.

The position reported by Schöpf and Becker (1936) for the main band in their absorption curve for xanthopterin is at a longer wavelength, λ 2750–2840 Å, than those reported for xanthopterin from other sources and by other workers mentioned above. Schöpf and Becker (1936) also reported that the absorption observed for some of their samples deviated slightly from that of their published curves and attributed this to the possible presence of degradation products in these samples.

It has been stated (Koschara, 1943) that xanthopterin seems to appear only in the animal kingdom, but the results of our studies of the tubercle bacillus, present and past (Crowe and Walker, 1949), indicate the synthesis by this organism of a pterin which in all of its chief characteristics closely parallels those of xanthopterin.

SUMMARY.

Studies of crystalline forms, fluorescence spectra, absorption in the ultraviolet and infra-red regions, and results of chemical tests are presented for purified samples of pterin derived from different strains of virulent human tubercle bacilli, grown in various media. All of the data closely conform to corresponding data reported for xanthopterin. It is concluded that this pigment, not previously reported for the human tubercle bacillus, is synthesized by this organism.

We wish to thank Dr. F. C. Nachod of Sterling-Winthrop Research Institute, Rensselaer, N.Y., for recording the infra-red absorption curves shown in Fig. 4.

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INVESTIGATION INTO THE PRODUCTION OF BACTERIOSTATIC SUBSTANCES BY FUNGI. PRELIMINARY EXAMINATION OF THE THIRTEENTH 100 SPECIES, ALL BASIDIOMYCETES.

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From St. Peter's Hall, Oxford.

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THE present paper which is the thirteenth in the series of papers under the above title, is the ninth which deals solely with Basidiomycetes. The fungi examined, which are all fungi of the wood-destroying type, were kindly sent to me by Dr. Mildred Nobles, Department of Agriculture, Ottawa. The names are those used by Dr. Nobles and the original laboratory numbers with an (N) are given after the names.

As in the previous paper (Wilkins, 1952), the fungi were grown on a peptone malt medium as well as on the usual malt and potato dextrose media. The metabolism solutions were tested against the standard bacteria *Bacterium coli* and *Staphylococcus aureus* by the Wilkins-Revised method (Wilkins, 1949). The results are recorded as " strongly positive," " weakly positive," and " negative," and, as in previous papers, only the S.P. are given in detail.

LIST OF FUNGI EXAMINED.

Strongly Positive.

	Result of test on liquid medium.			
			Zone diam. (mm.).	
	Time in days.	Medium*	*Bact. coli.*	*Staph. aureus.*
Polyporus borealis Fr. F. 3581(N)	45/65	PM	18	24
P. cinnabarinus (Jacq.) Fr. F1609(N)	40	M	—	22
P. palustris Berk. and Curt. 10620(N)	10	M	27	27
Poria monticola Murr. 11712(N)	30	PM	18	23
P. subacida (Peck) Sacc. 17780(N)	5	PM	—	25
P. vaillantii (Fr.) Cooke 17538(N)	15/55	M	20	23
P. xantha (Fr. ex Lind) Cooke 16570(N)	30	M	16	20

* PM = peptone malt ; M = malt.

Weakly Positive.

Hericeum laciniatum Leers ex Banker 21469(N) ; *Hydnum abietis* Hubert nomen nudum 17185(N) ; *Merulius americanus* Burt 17122(N) ; *Odontia bicolor* (Fr.) Bres. 21030(N) ; *Polyporus albolutens* (Ell. and Ev.) 16598(N) ; *P. betulinus* (Bull.) Fr. 9145(N) ; *P. dryophilus* var. *vulpinus* (Fr.) Overh. F830(N) ; *P. montanus* (Quél.) Ferry 11153(N) ; *P. obtusus* Berk. F6798(N) ; *P. radicatus* (Sow.) Fr. 17549(N) ; *P. subcartilagineus* Overh. 9417(N) ; *P. ursinus* Lloyd 16037 (N) ; *Poria carbonica* Overh. 8255(N) ; *P. eupora* (Karst.) Cooke 22250(N) ; *P. gilvescens* Bres. 21402(N) ; *P. mappa* Overh. and Lowe 21391(N).

Negative.

Hericeum (= *Hydnum*) *coralloides* Scop. ex S.F. Gray 17739(N); *H. erinaceus* (Fr.) Pers. 17672(N); *Hydnochaete olivaceum* (Schw.) Banker 17203(N); *Lentinus Kauffmanii* Smith 10722(N); *Odontia spathulata* (Schrad. ex Fr.) Litsch. 17751(N); *Odontia uda* (Fr.) Bres. 17754(N); *Polyporus abietinus* (Dicks.) Fr. F8012(N); *P. adustus* (Willd.) Fr. 17571(N); *P. albellus* Peck 21116(N); *P. amorphus* Fr. 21422(N); *P. anceps* Peck 17566(N); *P. balsameus* Peck 16128(N); *P. brumalis* (Pers.) Fr. 17033(N); *P. compactus* Overh. F8004(N); *P. cuticularis* (Bull.) Fr. 10194(N); *P. cutifractus* Murr. 16028(N); *P. delectans* Peck 21187(N); *P. dichrous* Fr. 22281(N); *P. distortus* (Schw.) Fr. F7992(N); *P. dryophilus* Berk. F2179(N); *P. elegans* (Bull.) Fr. 11360(N); *P. fragilus* Fr. 21387(N); *P. frondosus* (Dicks.)Fr. 10235(N); *P. fumidiceps* Atk. 21433(N); *P. fumosus* (Pers.) Fr. 11813(N); *P. galactinus* Berk. F3494(N); *P. gilvus* (Schw.) Fr. F1704(N); *P. guttulatus* Peck 9337(N); *P. hirsutus* (Wulf.) Fr. 21158(N); *P. hirtus* Quél. 16583(N); *P. hispidus* (Bull.) Fr. F2037(N); *P. melanopus* (Swartz) Fr. 22266(N); *P. pargamenus* Fr. 21149(N); *P. pubescens* (Schum.) Fr. 17578(N); *P. radicatus* Schw. 17521(N); *P. squamosus* (Huds.) Fr. 21167(N); *P. sulphureus* (Bull.) Fr. 17573(N); *P. tuberaster* Jacq. F984(N); *P. tulipiferae* (Schw.) Overh. 22262(N); *P. velutinus* (Pers.) Fr. 10238(N); *P. versicolor* (L.) Fr. 21150(N); *Poria aestivale* Overh. 21450(N); *P. albipellucida* Baxter 11692(N); *P. ambigua* Bres. 8448(N); *P. aurea* Peck 16609(N); *P. candidissima* (Schw.) Cooke 21448 (N); *P. cinerescens* (Bres.) Sacc. 17072(N); *P. corticola* (Fr.) Cooke 21435B(N); *P. crassa* (Karst.) Sacc. 21185(N); *P. crustulina* Bres. 22285(N); *P. decolorans* (Schw.) Cooke 21221(N); *P. ferrea* (Pers.) Bourd. and Galz. 9887(N); *P. ferrugineo-fusca* Karst. F1411(N); *P. ferruginosa* (Schrad. ex Fr.) Karst. 22257(N); *P. fimbriatella* (Peck) Sacc. 21407(N); *P. fissiliformis* Pilát ex Overh. and Lowe 21414(N); *P. griseo-alba* (Peck) Sacc. 22287(N); *P. incrassata* (Berk. and Curt.) Burt. 17819(N); *P. laevigata* (Fr.) Karst. 17586(N); *P. lenta* Overh. and Lowe 21206(N); *P. nigrescens* Bres. 21427(N); *P. notata* Overh. 21215(N); *P. obliqua* (Pers. ex Fr.) Bres. F7403(N); *P. oleracea* Davidson and Lombard 21454(N); *P. pannocincta* (Rom.) Lowe 21430(N); *P. prunicola* (Murr.) Sacc. and Trott 21451(N); *P. punctata* Fr. F1402(N); *P. semitincta* (Peck) Cooke 21224(N); *P. sericeomollis* (Rom.) Baxter 17779(N); *P. simulans* Overh. ined. 21395(N); *P. spissa* (Schw. ex Fr.) Cooke 21405(N); *P. tenuis* var. *pulchella* (Schw.) Lowe 21428(N); *P. tsugina* (Murr.) Sacc and Trott 11598(N); *P. undata* (Pers.) Bres. 21412(N); *P. unita* (Pers.) Karst. 21210(N); *P. versipora* (Pers.) Rom. 21453(N); *P. Weirii* Murr. 10152(N).

Of the above fungi ninety fall into two almost equal numbers of species of Poria and Polyporus and a comparison between the two genera shows striking similarity in antibacterial activity.

	Poria.	Polyporus.	Total.	
SP.	4	3	7	8 per cent
WP.	4	8	12	13 per cent
N.	36	35	71	79 per cent
	44	46	90	

In general, the results do not support the opinion that wood-destroying fungi tend to have a high positive/negative ratio nor the previous statement by the

author (Wilkins, 1950) that the expected positive/negative ratio was approximately 50 per cent. The truth of the statement has already been questioned, but whereas it was previously attributed to loss of fungal activity consequent upon a prolonged period of time in culture, it is now very tentatively suggested that it may also be due to increased bacterial resistance.

The total time during which the metabolism solution was tested was again 45 days. In some cases this was increased to 60 days and 95 days respectively but it was confirmed that the longer time gave no significant advantage.

Though the fungi were grown on three media it will be noted that only two media, *viz.*, malt and peptone malt, are given in the S.P. list. The problem of choosing a medium which is favourable for any given fungus is a difficult one, but that of choosing a medium which is equally favourable for the production of an antibacterial metabolism solution by a wide range of fungi is, in our experience, impossible. Experiments over a period of ten years have shown that a few media are favourable for most fungi, and it has been found that for Basidiomycetes these could be reduced to two, *viz.*, potato dextrose and malt. Carlier (1948) found that a medium consisting of 4 per cent dextrose and 1 per cent " Oxoid " peptone, with agar and water in the usual proportions, was very suitable for the growth of certain pathogenic fungi. We had recognised that peptone, particularly " Oxoid " brand peptone, was a valuable constituent of mycological media and with the assistance of Miss June Rees, an investigation into the suitability of the Carlier medium in a liquid form for the production of metabolism solution by Basidiomycetes was started. The tests were made with *Polyporus betulinus* W620B, which had the merit of growing rapidly and producing from the media M and PD metabolism solutions which were strongly bacteriostatic against both *Bact. coli* and *Staph. aureus*. On the Carlier medium the fungus produced peaks of antibacterial activity, which were higher and which persisted longer than on either M or PD. It was found, however, that the medium itself was bacteriostatic due to the high proportion of dextrose and consequent liberation of hydrogen peroxide. Media containing different quantities and proportions of malt, dextrose and peptone were tested. The metabolism solution produced by W620B was strongly positive but in all but three cases, when the percentage of dextrose was less than 1 per cent the medium itself was bacteriostatic. The only media which merited further trial were two : peptone 1 per cent, dextrose 1 per cent and peptone 1 per cent, malt 5 per cent. Neither was bacteriostatic. The former supported a vigorous fungus growth for about 35 days at which point the mycelium fragmented and there was no further growth. Incidentally, the fact that disintegration of the mycelium resulted in an immediate increase in zone size suggested that an antibacterial substance was released from the mycelium at this stage. From these experiments it was found that the only essentially favourable medium incorporating peptone was peptone malt (PM), consisting of " Oxoid " peptone 1 per cent, malt 5 per cent. This medium was the one used in the above work and it justified its inclusion in three ways : (1) It was a satisfactory medium in which to grow isolates from living fungi of all sorts. (2) Growth in solid media was good and " strip-test " zones were relatively large. (3) Metabolism solution zones were also satisfactory. It will be noted in the list of SP results that the medium PD is not included as in every case the results were better on either of the media M or PM.

Since the author's last publication Bose (1952), testing certain Basidiomycetes

for antibacterial activity by the author's revised testing method, found that the most promising results were obtained from *Psalliota campestris*. Though tested many times in this laboratory this species consistently failed to give a positive result. As the testing method was common to both workers, the success might possibly be attributed to the medium used by Dr. Bose, a medium not generally favoured by us but which may be specifically favourable for this species of *Psalliota*.

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THE SELECTIVE ABSORPTION OF HAEMOLYTIC COMPLEMENTS BY ANTIBODIES FROM DIFFERENT SPECIES OF ANIMALS.

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In a previous study (Blomfield, Coombs and Hole, 1949) the haemolytic complement of the guinea-pig was compared with the conglutinating complements of the horse, pig and cat for the ability to be absorbed by antibodies from different species of animal. The absorption of the complement of the guinea-pig was found to be inferior to that of the horse.

Although sera from animals other than the guinea-pig such as the rabbit and man which contain haemolytic complements, are easily available in the laboratory, the guinea-pig is the animal which has the highest titre of haemolytic complement. In the method of titration which we use 1 M.C.D. of complement is usually contained in a unit volume containing a 1/160 dilution of guinea-pig serum, a 1/40 dilution of human serum and a 1/10 dilution of rabbit serum.

However, the strength of the haemolytic activity of the sera is probably quite unrelated to the absorbability of the complements. It was in order to compare the absorbability of the haemolytic complements of the guinea-pig, rabbit and man by antibodies from different species of animals that the present experiments were performed. From the results it is clear that although the haemolytic complement titre of rabbit serum is very low, nevertheless this complement is in nearly all cases the most absorbable of those tested.

METHODS AND MATERIALS.

Complement fixation.—Anti-mallein sera produced in different animal species were examined by the haemolytic complement fixation test for their content of complement-fixing antibodies. The method used in these tests has been described by Hole and Coombs (1947) and Coombs and Hole (1948).

Comparable tests were performed using 2 minimal complement doses (M.C.D.) of guinea-pig, rabbit and human complements.

The period allowed for the fixation of complement was 1 hr. at room temperature before the addition of the haemolytic indicator system.

Antisera.—The anti-mallein sera were produced as described in our previous paper (Blomfield *et al.*, 1949). All the sera were obtained after a single course of inoculations except the human serum which was a second course serum. Many of the sera had been stored at −20° for 3–4 years before testing. Before use they were inactivated at 56° for half an hour.

Antigen.—The mallein antigen was prepared as described by Coombs and Hole (1948) and used at its optimal dilution.

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All the sera were tested once using the antigen diluted 1/700. A year later they were retested using the antigen 1/500 and practically identical results were obtained.

Complements.—Unheated sera of guinea-pigs, rabbits and man were used. They were stored at −20° without the addition of a chemical preservative.

Indicator system.—This consisted of equal parts of a 1 per cent suspension of sheep red cells and a 1/400 dilution of horse anti-sheep red cell haemolysin representing three minimal complete haemolytic doses.

RESULTS.

The titres of antibody to mallein demonstrated in the different antisera by means of the three haemolytic complements are recorded in the Table.

Anticomplementary activity of sera was evident only in the sera of pigs and dogs and of a ferret and this activity, unfortunately, was directed solely against rabbit complement.

TABLE—*Titres of Antibody to Mallein in Antisera from Different Animal Species with Three Haemolytic Complements.*

Anti-mallein sera.	Titre of antibody detected by absorption of 2 M.C.D. of the complement of :		
	Guinea-pig.	Rabbit.	Man.
Human C	5	80	5
Horse 1	1280	1280	40
„ 10	1280	1280	160
Guinea-pig (pool)	5	160	0
„ 1639	20	320	5
Rabbit 1801	160	160	40
„ 1802	320	640	80
Cat O	0	80	0
„ T	(5)	160	40
Ox 1000	40	640	40
„ 1019	20	640	40
Sheep 1921	80	320	80
„ 1941	320	2560	320
Goat 1231	80	320	160
„ 1295	40	320	80
Ferret 1149	5	1280 [20]	40
Pig C 1	0	80 [40]	10
„ C 2	0	80 [40]	5
Dog L	20	640 [40]	40
„ R	40	640 [40]	80
Fowl 5	0	0	0

0 = <5.
(5) = doubtful reaction at 1/5.
[40] = anticomplimentary activity of serum alone at 1/40.

All the pre-inoculation sera were examined and found to be negative as regards antibody to mallein except the sera of the sheep and goats which showed

with both rabbit and human complement an apparent low natural titre (<10). This low level of antibody was also demonstrated using horse complement and a conglutinating system, and was also shown in the sera of a further nine normal sheep and five normal goats.

Fowl antibodies, which are known not to fix guinea-pig complement, also failed to absorb rabbit and human complement.

DISCUSSION.

Factors other than the haemolytic titre of a complement have to be considered when choosing the complement to use in a haemolytic complement fixation test. In the immune system investigated in this work, namely mallein-anti-mallein with the employment of 1 hour for fixation at room temperature, guinea-pig complement was by no means the most sensitive in demonstrating antibodies whilst rabbit complement showed that it possessed great potentialities.

In other antigen-antibody systems guinea-pig complement may well show to better advantage and it is known that overnight fixation at 4° increases the amount of absorption. Whether the absorption of rabbit complement is increased by overnight fixation has not been tested.

Although rabbit complement offers promising potentialities, it is possible that being very sensitive to absorption by antigen-antibody complexes it may also be very susceptible to what are called non-specific anti-complementary factors. Indeed in this work the pig and dog sera and a single ferret serum were anti-complementary towards it. A thorough study of the mode of action of anti-complementary factors in both sera and antigen preparations could probably show a way of overcoming them, while at the same time not interfering with the specific absorption by antigen-antibody complexes. After such a study the merits of rabbit serum as a complement for use in the haemolytic complement fixation test should be further appraised.

SUMMARY.

Anti-mallein sera from 11 species of animal have been examined for their titre of complement fixing-antibody using 2 M.C.D. of guinea-pig, rabbit and human haemolytic complements. A comparison of the results showed that, with the method used for fixation, the complement of the rabbit was the most sensitive in demonstrating the antibody in nearly all the sera.

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THE MECHANICS OF SUBCUTANEOUS INJECTION.

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WHEN single-cell suspensions of transplantable mouse sarcoma are injected subcutaneously into mice, an inoculum of 0·2 ml. gives rise, immediately after injection, to a palpable swelling which measures about 1 cm. across. The tumour which results from such an inoculum, however, appears to arise from a single focus very much smaller than the infiltrated area. Craigie (1949), discussing various technical considerations relating to the quantitative transplantation of tumours, was unable to explain this disparity between the distribution of the inoculum and the minute singular origin of the resulting tumour.

Investigations of the dispersion of various fluid preparations immediately after subcutaneous inoculation have provided a satisfactory explanation for this anomaly. The results of these investigations are reported here because they may be of significance in a variety of experiments in which subcutaneous inoculation, especially of suspended matter, forms part of the procedure. Although the findings to be described are easily predictable from an elementary knowledge of the structure of loose connective tissue, they do not appear to be widely realised.

MATERIALS AND METHODS.

Albino mice were anaesthetised by the intraperitoneal injection of avertin, and pinned down with the ventral surface uppermost. A ventral cruciform incision was made through the skin and subcutaneous tissue and the potential four large triangular flaps were freed from the underlying muscle by traction. The flaps so made were seen to have an intact blood supply from the axillary or groin regions.

Injections were made with a tuberculin syringe carrying a Schick test needle. With the needle parallel to the skin surface, a thin tent of connective tissue was picked up on the needle point from the under surface of a flap; the needle was then advanced about 1 cm. into the tissue and the injection made fairly slowly. The use of coloured preparations permitted direct observation of the dispersion of the material in the tissues during the injection. It was found that the appearance of an inoculum placed in this way did not differ from that of one injected subcutaneously through the skin in the usual way.

The preparations injected were as follows:—

(1) A suspension of carmine in liquid paraffin.

(2) The supernatant fluid of a 0·05 per cent solution of trypan blue in saline which had been centrifuged at 3000 r.p.m. for 10 min. to remove undissolved particles of dye.

(3) Colloidal gold solution containing 0·16 per cent sodium citrate, the pH of the final solution being 5·9.

(4) India ink diluted 1/10,000 in saline; this suspension was hardly distinguishable from water when seen in a test tube.

(5) A suspension of carmine in saline, prepared as follows: a 10 per cent ground suspension of the dye in saline was centrifuged at 800 r.p.m. for 10 min. and the deposit discarded; the supernatant fluid was centrifuged at 6000 r.p.m. for 15 min., and the clear pink supernatant fluid discarded; the deposit was washed in saline and finally re-suspended; the suspended particles of dye

were found to be of about the size of cocci, and their concentration was such as to give a pink opalescent fluid.

(6) A suspension in saline of *Bact. coli* which had been washed free of medium, stained with methyl violet and Gram's iodine, and finally washed free of uncombined dye. The suspension was centrifuged at 800 r.p.m. for 10 min. to remove aggregates of bacteria.

(7) Untreated cows' milk containing 0·01 per cent trypan plue added in saline solution.

(8) A suspension of Krebs carcinoma ascitic cells in Tyrode solution.

In every experiment, an injection of any of these preparations was in a total volume of 0·2 ml.

In some experiments the mice had been pre-treated with " Hyalase " (Benger): about 200 units of " Hyalase " were injected subcutaneously in a region close to that used for the tests. When the " Hyalase " was given one hour before injection of any of the other preparations, its effects extended over a fairly wide area surrounding the original area of infiltration.

EXPERIMENTS AND RESULTS.

Carmine suspension in liquid paraffin.

Injections of this preparation resulted in homogeneous red hemispherical protuberances of about 1 cm. diameter. When a swelling was bisected with a scalpel the contents were freely discharged. There was no separation of the carmine and oil. Since the oil cannot diffuse into the tissue it is not dispersed but remains as a cyst. The space which it occupies is created by mechanical distention of the small potential space created by the trauma of the needle. Although additional trauma may result from the hydrostatic pressure produced during injection, some of the distention is exerted against an elastic force in the tissue tending to close the cavity; this last factor is indicated by a tendency for the oil to be expelled back through the needle track.

Trypan blue in saline.

An inoculum of 0·2 ml. of a 0·05 per cent solution of this dye in saline, when examined immediately after injection, forms a homogeneous blue hemispherical swelling over 1 cm. in diameter. The size of the swelling showed very little tendency to increase over the one hour period following its formation. No concentration of the dye was seen in the vicinity of the needle point. Digital compression of the swelling immediately after injection did not result in the expression of any appreciable quantity of dye along the needle track. When the summit of the swelling was touched with a filter paper, it was shown that blue dye was exuding through the wall. When the swelling was bisected with a scalpel within a few minutes of its production, very little blue fluid was freed, and two discrete blue swellings resulted. When the injection was made into " Hyalase "-treated tissue, the initial swelling dispersed almost as soon as it was formed and blue fluid flowed freely from its surface. With diffusible dyes, it appears that the area of palpable infiltration is a rough measure of the initial distribution of the injected dye.

Suspensions in saline of carmine and of stained bacteria.

The dye particles in carmine suspensions prepared as described are of about the same size as cocci. Since the findings were essentially the same for carmine and stained bacterial preparations, only those obtained with carmine will be described.

During injection of the 0·2 ml. volume a dense red speck appeared at the needle point almost as soon as the injection was begun. Though growing in size as the injection proceeded, the dense red spot remained very small compared with the size of the oedematous tissue which surrounded it. When more concentrated suspensions of carmine were used the red zone remained quite localised, and the surrounding swelling was quite colourless. It was found that the localised spot of dye often appeared larger than it actually was owing to the convex oedematous tissue surrounding it acting as a magnifying lens. This effect was abolished by viewing the dye spot through a glass slide pressed on the swelling to give it a plane surface. When the swelling was frozen at $-70°$ and incised in various planes perpendicular to the skin surface, examination of the cut surfaces revealed that the localisation and concentration of the dye could be appreciated from all viewpoints. Generally, the carmine mass did not exceed 2 mm. in any diameter.

Fig. 1 shows, side by side, the appearances of trypan blue solution (A) and of carmine suspension (B), after injection into the exposed subcutaneous tissue; both were in a volume of 0·2 ml., and the extent of the trypan blue may be taken as an indication of the size of the oedematous zone surrounding the carmine spot.

Concentration of dye at the needle point occurred in precisely the same manner when the injection was made into "Hyalase"-treated tissue, the only difference being that the surrounding oedema dispersed very much more rapidly.

India ink and colloidal gold.

The behaviour of India ink resembled that of carmine suspension rather than of trypan blue solution. Although there was marked concentration of the ink at the needle point, the concentrated focus had a slightly more indefinite edge than the carmine focus and appeared to extend rather further into the surrounding oedematous zone.

Colloidal gold showed little or no concentration at the needle point, the red colouration of the tissue being co-extensive with the area of visible oedema. There was, however, a distinct graduation of the colour from the needle point to the peripheral zones; it is possible that this reflects a gradation of size in the particles constituting the colloid.

Milk.

Milk containing trypan blue was used to study the behaviour of a typical oil-in-water emulsion. The trypan blue, soluble in water but not in fat, was used as a differential stain to distinguish the continuous phase from the disperse phase. After injection of 0·2 ml., the blue protuberance produced was opaque. It was therefore necessary to bisect the swelling and examine the cut surface in order to study the distribution of the injected matter. The cut surface revealed an opalescent blue area containing a central pale zone from which the blue colour had been almost completely excluded. This central zone evidently represented a localised condensation of the discontinuous oil phase; injection had resulted in separation of the cream. The opalescence of the more diffused part of the injected area is assumed to be due to the caseinogen present in colloidal solution

in the continuous phase of the milk. It is known that the white opacity of milk is only partly due to the fine fat globules (Hawk, Oser and Summerson, 1949).

Ascitic tumour cells.

A suspension of the ascitic cells was diluted with carmine suspension to give a mixture in which the carcinoma cells occupied < 2 per cent of the total fluid volume. When 0·2 ml. of the mixed suspension was injected, the concentrated carmine was observed at the needle point and was used as a marker to indicate the probable situation of the carcinoma cells in the tissue. The area of skin and subcutaneous tissue which was involved in the swelling was excised after one hour and fixed in Bouin's solution. The tissue was prepared for histological examination and serial sections were made of the zone containing the carmine. The appearances seen in a typical section stained with haematoxylin and eosin are shown in Fig. 2. It will be seen that the tumour cells are densely packed together, forming a solid plaque measuring about 2 mm. in diameter; no connective tissue fibres are seen among the cells and no isolated tumour cells are seen in the surrounding connective tissue. The appearance is hardly distinguishable from that of an area of solid tumour. It is evident that the cells have not been distributed through the tissue along with the fluid medium in which they were suspended. It has been found that injections of more dilute suspensions of cells do not give rise to a compact mass of cells. This is to be expected, since the cells are then distributed over the volume of tissue traumatised by the needle and are insufficient to fill it. In these circumstances the cells lie singly or in small groups within the traumatised connective tissue.

It has been observed that sites which have been injected with mixed suspensions of tumour cells and carmine reveal that the dispersion of the carmine is slightly wider than that of the cells but only in a plane parallel to the skin surface.

DISCUSSION.

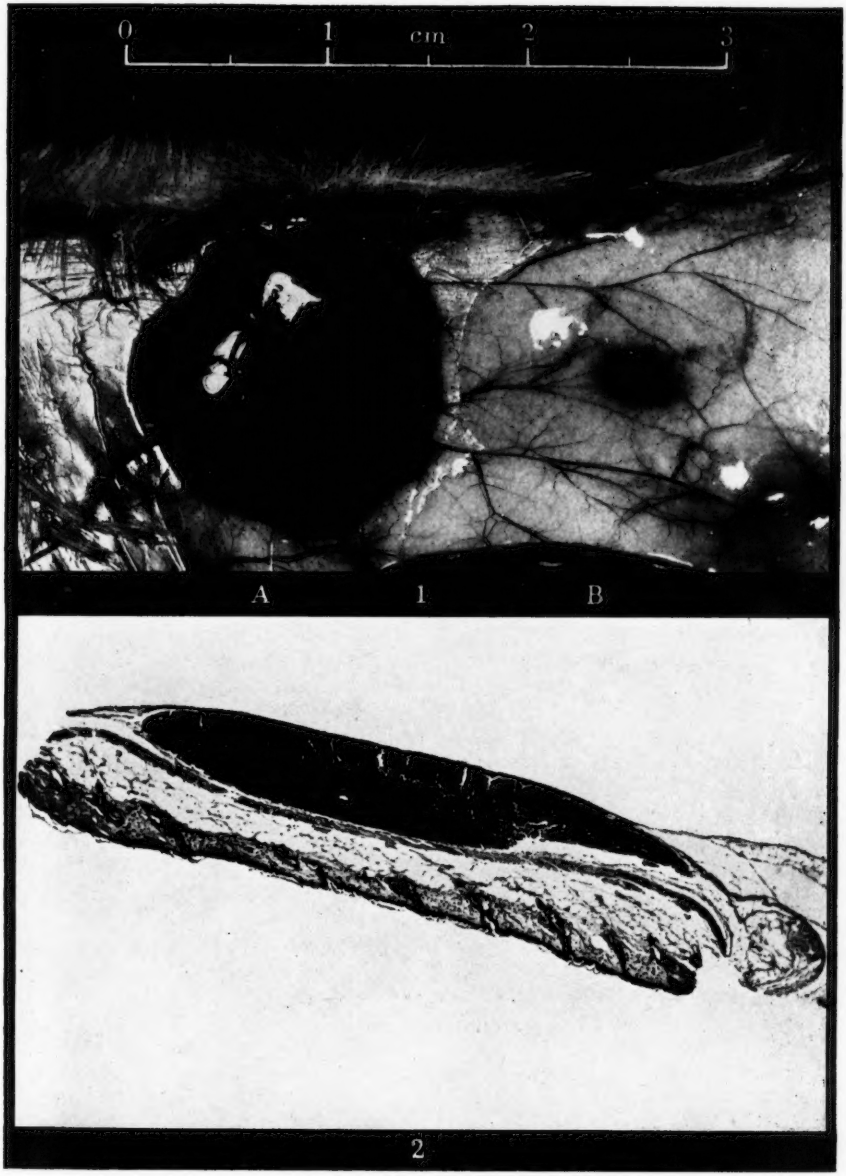
Loose connective tissue is described as consisting of a homogeneous ground substance in which are embedded a close meshwork of collagen and elastin fibres, and isolated cells. The fibres of elastin are not numerous and the tissue as a whole would not be expected to be very elastic (Maximow and Bloom, 1942). A simple model of comparable, though coarser and tougher, structure would be a Seitz filter disc soaked in a soft gel. The observations which have been reported are readily explicable from mechanical considerations which follow from the structure of connective tissue so visualised.

Oils or any other liquids which form an interface with aqueous media are unable to diffuse either into the gelatinous ground substance or into the collagen mesh. The injected fluid therefore remains as a globule which enlarges as further fluid is injected. The hydrostatic pressure created by the advancing piston of the syringe is transferred to the walls of the enlarging globule and this produces traumatic splitting of the connective tissue in accordance with the space require-

DESCRIPTION OF PLATES.

FIG. 1.—Appearance in the subcutaneous tissue of (A) 0·2 ml. of trypan blue solution; (B) 0·2 ml. of carmine suspension. × 3.

FIG. 2.—A subcutaneous inoculum of mouse ascites tumour cells injected as a single-cell suspension in a volume of 0·2 ml. ×43.



Hewitt.

ments of the inoculum. The injection of air apparently follows a similar course ; the multiple loculation which occurs in surgical emphysema is probably due not so much to the existence of potential spaces in the tissue as to the presence of thick bands of collagen fibres which are left behind when weaker bands are disrupted. The localisation of small particles here described does not suggest the presence of intercommunicating spaces in undamaged tissue such as is often postulated to explain the bubbly appearance of emphysema, and from which the term " areolar " is derived.

The localisation of dye particles, bacteria, and tumour cells suspended in saline is easily understood when the following three reasonable assumptions are made : (1) that the aqueous suspending medium diffuses readily through untraumatised connective tissue, (2) that the particulate matter will not pass through either the intact ground substance or the collagen mesh, (3) that the hydrostatic pressure created by the advancing piston of the syringe is dissipated in diffusion of the medium before it can inflict trauma additional to that already brought about by the needle during its insertion. Under these conditions the particulate matter will remain in the traumatised zone immediately adjacent to the needle point while the suspending medium will diffuse out into the surrounding tissue. The process is essentially one of filtration. Only when the total volume of non-diffusible matter exceeds the space created by the trauma due to the needle does further trauma result.

The fact that " Hyalase " has no appreciable effect on the localisation and concentration of particles indicates that the collagen mesh alone is sufficient to filter out the particles. The only effect of the hyaluronate ground substance appears to be to slow the rate of diffusion of the diffusible part of the inoculum. The ease with which inflammatory cells pass through the connective tissue under a chemotactic influence indicates that the filtration effect described may depend more upon adsorption than upon mechanical obstruction.

From the behaviour of milk, it appears that injection of oily substances in the form of emulsions does not aid absorption of the oil by widening the distribution of the material. The oil globules of the cream which separates from milk during injection undergo progressive coalescence. This is to be expected if the emulsifying agents are diffusible, as they would then be removed from the system by diffusion into the surrounding tissue with consequent breakdown of the emulsion.

In view of the observed localisation of subcutaneously injected India ink, it is reasonable to suppose that the larger viruses certainly and the smaller viruses most probably, would be deposited in a very small locus when suspensions are so injected. There is some evidence that the initial distribution of an immunising antigen in the tissues may influence its effectiveness. Immunity induced by vaccination, for example, has been found to be greater after multiple insertions than after a single insertion (Report, 1928). It may thus be surmised that the inoculation of particulate antigens (viruses or bacteria) by propulsion through the skin in a powerful pneumatic jet, a method for mass inoculation which has been under trial in the United States, might give greater immunity than the inoculation of equivalent doses by hypodermic needle.

The results reported provide an adequate explanation of the observation that the tumours resulting from inoculated tumour cell suspensions arise from a single focus very much smaller than the infiltrated area produced. The fact

that inoculation brings the cells closer together than they are in the suspension before injection may be of some importance to an understanding of the factors which determine the minimum number of cells required to transplant a tumour. Koller (1947) describes a human adenocarcinoma in which synchronous mitosis of adjacent cells was a particular feature, and refers to evidence from various sources which suggests that the cells of some tumours may be dependent upon one another for a stimulus to divide. It is in this connection that alterations of the spatial distribution of tumour cells on inoculation might prove to be significant.

It is sometimes desired to obtain the cells of ascitic tumours in a compact mass so that they may be prepared and examined by the histological methods employed for blocks of solid tissue. Centrifugation, which usually forms part of any such procedure, has the disadvantage of producing an artificial distribution of the cells owing to variation in the rate of sedimentation of the different cell types. Difficulty may also be experienced in removing the compact cell deposit from the tube. A simple method of obtaining ascitic cells in a compact mass without these difficulties is to inoculate into the exposed subcutaneous tissue a mixture of the cell suspension with dilute carmine suspension and " Hyalase " solution. After about one hour the local oedema has dispersed and the carmine marks the compact cell mass. The piece of tissue containing the marker can then be excised, fixed, and blocked. Serial sections enable the ascitic cells to be examined as though they constituted a piece of solid tumour.

SUMMARY.

The distribution of various coloured fluids and suspensions immediately after injection into loose connective tissue has been studied under direct observation by making the injections into the undersurface of retroverted skin flaps in living mice.

When suspensions of particulate matter are injected, the particles (stained bacteria, India ink, carmine, tumour cells) do not diffuse far from the needle point but are concentrated and localised at this site while the suspending medium diffuses outward.

Injection of an emulsion results in immediate separation of the discontinuous from the continuous phase. The dependence of these " filtration " effects on the accepted structure of connective tissue is described, and several implications of the findings are discussed.

I am indebted to the British Empire Cancer Campaign for grants during the tenure of which these investigations were made, to Miss Catherine Fysh, B.Sc., for skilled technical assistance, and to Dr. P. Hansell and the Department of Medical Photography, Westminster Medical School, for the photographs.

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A CONTRIBUTION TO THE NATURE OF THE ELASTOLYTIC ENZYME AND THE STRUCTURE OF ELASTIN.

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THE presence of a bacterial elastolytic enzyme was discovered as early as 1904 by Eijkman. He prepared culture media from the lungs of calves, from ligamentum nuchae and from arteries. With these he demonstrated the lysis of elastin as the result of bacterial action. Of the bacteria that he studied *Ps. pyocyanea* showed the highest activity. In 1949 Baló and Banga reported that extracts of fresh ox pancreas as well as that of acetone-dried pancreas powder contain a specific elastolytic enzyme which they named elastase. In further publications Baló and associates demonstrated that the enzyme does not attack collagen and that their pure elastase differs from the other proteolytic ferments of the pancreas in that neither trypsin nor chymotrypsin have any comparable dissolving action on elastic tissue (Baló and Banga, 1950; Banga, Baló and Nowotny, 1948–49; Banga, 1951). Banga (1951) suggested that the effect of elastase was not proteolysis, but rather depolymerization, and that this was effected by the splitting of H-bonds, fibrous elastin being transformed into globular elastin.

The next important advance in our knowledge of the nature of elastin was taken when Hall, Reed and Tunbridge (1952) observed that polysaccharide and sulphuric acid are intimately associated with the protein in elastic tissue. In their view elastic tissue consists of linear aggregates of pro-elastin protected on the outer side by a layer of pro-elastin molecules bound together by union with polysaccharide molecules. In support of this they found that during the action of elastase on elastin all the acid is liberated during the dissolution of only half the protein. Furthermore, they also expressed doubt as to the function of elastase and suggested that it is a mucase rather than a proteinase. According to them the function of the enzyme would be to attack the elasto-mucin chains, liberating the pro-elastin therefrom.

These observations of Hall *et al.* were further substantiated by Banga and Schuler (1953). From their chemical experiments, they concluded that elastin was a glucoprotein, the fibrous character of which is determined by the carbohydrate molecule of elastin. Following dissolution of elastin by elastase, they demonstrated a carbohydrate molecule in the deproteinized filtrate.

Neuberg and Hoffmann (1931) demonstrated the presence of a chondrosulphatase by obtaining a specific enzyme preparation from an organism related to *Ps. eisenbergii*. These authors also found chondrosulphatase in *Proteus vulgaris* and *Ps. pyocyanea*. This, taken in conjunction with the earlier work of Eijkman (1903–04) would suggest that extracts of *P. vulgaris* contain a chondro-

sulphatase as well as an elastolytic enzyme. Our own observations as regards pancreatic elastase and the bacterial chondrosulphatase and/or elastase have led us to believe that they are identical enzymes, attacking primarily the mucopolysaccharide bonds between the pro-elastin molecules.

MATERIALS AND METHODS.

Preparation of pancreatic elastase.

Pancreas powder was prepared according to the method of Willstätter and Waldschmidt-Leitz (1923). The fine powder was stored in a glass-stoppered bottle in a refrigerator at 4°. In all the experiments a 1 per cent solution of this powder in distilled water was used. The solution was put into a Coplin jar containing the deparaffinized sections to be digested and incubated at 37° for varying periods of time. The solution of the enzyme remained stable for 2–3 days. The pH of a freshly prepared aqueous solution of the enzyme as determined with a Beckman pH meter was 8·8. After incubation for 2 hr. at 37° the pH was found to be 6·6. Although the optimum pH for elastase is given as 10·3, we found that buffering of the solution was not necessary as the enzyme activity on tissue sections was not significantly affected at this pH. Control sections were incubated at 37° in distilled water.

In order to see whether the pH change during incubation would have any effect on the staining reactions used, sections of aorta and costal cartilage were incubated at 37° for 6 hr. in McIlvaine Na_2HPO_4-citric acid buffer solutions in which the pH range varied from 2·2–8·0. No interference was found in the subsequent staining of the aortic elastic tissue with Weigert's or Verhoeff's elastic tissue stains or of the metachromatic material in the aortic wall and cartilage on staining with a watery solution of toluidine blue.

Preparation of chondrosulphatase.

A 48-hr. nutrient agar growth of *P. vulgaris* and *Ps. pyocyanea* was suspended in acetone, allowed to stand for 2 hr. and then washed in an ether-acetone mixture and finally left in pure anaesthetic ether for another 2 hr. The suspension was then filtered, and the bacteria left to dry on filter-paper overnight. The powder so obtained was then ground with a pestle and mortar. A 2 per cent solution of this powder in distilled water was used in the subsequent experiments. Sections of aorta and cartilage were deparaffinized and incubated in the solution contained in Coplin jars for varying periods of time. Control sections were incubated in distilled water at 37° for the same periods. Where sections were incubated for periods exceeding 6 hr. it was found useful to cover the slides with a thin layer of celloidin to prevent the tissue lifting from the slide. In addition the hyaluronidase and trypsin extractions were done according to the methods suggested by Pearse (1953).

Substrates.

The aorta and costal cartilage used in this experiment were fixed in 10 per cent formol-saline, dehydrated in alcohol and embedded in paraffin wax. Sections were cut at 4μ, dewaxed and subjected to the action of the different enzymes.

The metachromatic substance of the aorta is composed of the acid mucopolysaccharides chondroitin sulphate B and C, whereas that of hyaline cartilage consists of chondroitin sulphate A (Meyer and Rapport, 1951). From a histological point of view elastic tissue is characterized by a specific staining reaction. The exact nature of the reaction of these different stains is not known. Chemically elastin differs from collagen in having less arginine, histidine and lysine and more leucine, valine and tyrosine. No tryptophan is present in elastin. Davies and Dubos (1947) have shown that leucine residues are lipophilic and that elastic tissue might therefore contain more lipid than collagen. In addition it has recently been shown that elastic tissue contains a carbohydrate.

Staining methods.

The staining methods used to demonstrate elastic tissue were Weigert's elastic tissue stain, prepared from basic fuchsin and resorcin, and Verhoeff's haematoxylin method. The metachromatic substance was stained by aqueous toluidine blue (Pearse, 1953). Masson's

trichrome stain and periodic-acid Schiff reactions were used to differentiate collagen and fibrous tissue.

The results are presented in the Table.

DISCUSSION.

The following two observations detailed in the Table led us to suspect that the enzyme prepared from ox pancreas is similar to the bacterial chondrosulphatase.

Firstly, both these enzymes, when allowed to react for a short period with the chondroitin sulphate present in the wall of the aorta and costal cartilage, cause disappearance of this acid mucopolysaccharide as shown by the absence of metachromatic staining with a watery solution of toluidine blue.

Secondly, when these enzymes are left in contact with the elastic tissue of the aorta for a longer period, they both cause elastolysis. The proof that elastolysis had actually taken place and that the failure of elastic lamellae to stain with the usual stains was not due to a chemical reaction of the enzyme with elastin, has been proved by the absence of such lamellae on examination of sections after extraction by means of phase-contrast microscopy.

These observations would then suggest that pancreatic elastase is in reality a chondrosulphatase and would lend support to the work of Baló and Banga, and Hall *et al.* in explaining the chemical nature of the reaction of the enzyme. Removal of the binding polysaccharide molecules in the elastin chain would then result in the setting free of pro-elastin molecules. Free amino-acids would not be set free in this reaction.

The pancreatic extract used in this experiment contains in addition to elastase other enzymes, the most important of which is trypsin. As the pH of the 1 per cent solution of pancreatic extract varied between 8·8 and 6·6 during incubation, it was necessary to do control histochemical experiments with crystalline trypsin, as this enzyme has its optimum effect at pH 8·0. It was found, however, that pure crystalline trypsin had no elastolytic effect, and furthermore that the amount of trypsin in the 1 per cent solution of pancreatic extract was relatively small, the collagen in the aortic wall not being significantly affected after incubation for 6 hours.

It was also noted that the elastolytic enzymes removed the free acid mucopolysaccharides before attacking elastic tissue itself and therefore the possibility that it might be a hyaluronidase had to be investigated. Using testicular hyaluronidase according to the method described by Pearse it was found that it removed the free mucopolysaccharides of both cartilage and aorta, but the elastic tissue was unaffected after prolonged incubation.

The fact that elastic tissue contains a chondroitin sulphate is important in so far that the age increase of mucopolysaccharide in the aorta might be due to degeneration of elastic tissue and liberation of the acid mucopolysaccharide therefrom. Many theories have been advanced as regards the origin of the mucopolysaccharides of the aorta but as yet none of these has been definitely proved. In none of these has an origin in elastic tissue been suggested. Schultz (1922), however, suggested that the metachromatic substances might furnish material for the building up of elastin and that chondroitin might be among them.

A further interesting observation has been that staining of elastin before

digestion with the enzyme inhibits the latter action. In addition it is also well known that cartilage gives a false positive staining reaction with the usual elastic tissue stains. These two observations would suggest that the specificity of the elastic tissue stains is possibly dependent on the presence of the acid-mucopolysaccharide in elastin. Schultz (1922) suggested that the staining reaction of elastic tissue by the elastic stains is due to the impregnation of elastin by mucin.

TABLE.—*Comparison of Results.*

Substrate.	Enzyme.	Time of reaction in hours.	Staining method.	Results.
A.M.P.A.	Pancreatic elastase	1	Toluidine blue	Total removal of metachromatic material.
,,	Hyalase	3	,, ,,	Total removal of metachromatic material.
,,	Trypsin	1	,, ,,	Normal amount of metachromatic material.
,,	Bacterial chondrosulphatase	2	,, ,,	Total removal of metachromatic material.
A.M.P.C.	Pancreatic elastase	½	,, ,,	Normal amount of metachromatic material present.
,,	,, ,,	¾	,, ,,	Small amount of metachromatic material still present.
,,	,, ,,	1	,, ,,	Total removal of metachromatic material.
,,	Hyalase	3	,, ,,	Total removal of metachromatic material.
,,	Trypsin	1	,, ,,	Normal amount of metachromatic material present.
,,	Chondrosulphatase	1	,, ,,	Total removal of metachromatic material.
Elastin (aorta)	Pancreatic elastase	1	Weigert	No effect.
,, ,,	,, ,,	2	,,	Fibres and lamellae begin to swell and early fragmentation can be seen.
,, ,,	,, ,,	3	,,	Patchy disappearance of fibres. Remaining fibres have moth-eaten appearance.
,, ,,	,, ,,	4	,,	Occasional poorly stained fibres remain.
,, ,,	,, ,,	5–6	,,	Total removal of elastin.
Elastin	Hyalase	6	,,	Normal amount of elastin present.
,,	Trypsin	6	,,	Normal amount of elastin present.
,,	Chondrosulphatase	6	,,	Total removal of elastin.
Aorta	Pancreatic elastase	6	Masson	Collagen still present.
Elastin first stained with Weigert	,, ,,	6	Weigert	Normal amount of elastin still present.
Elastin extracted in chloroform methanol for 6 hr.	None	6	Weigert PAS Toluidine blue	Normal staining reaction.
Elastin (aorta)	Pancreatic elastase Chondrosulphatase	6	None	No elastic lamellae seen by phase contrast microscopy.

A.M.P.A. = Acid mucopolysaccharide of aorta.
A.M.P.C. = ,, ,, of cartilage.

Further morphological, histochemical and experimental studies are being carried out to determine the relationship between the break down of elastic tissue, the increase of metachromatic ground substance and the ultimate collagenization in the pathogenesis of the degenerative arterial diseases.

SUMMARY.

Specific elastase prepared from ox pancreas and bacterial chondrosulphatase obtained from *Ps. pyocyanea* and *P. vulgaris* appear to be identical enzymes.

When these enzymes act on tissue sections for a short time, they have a chondrosulpholytic effect, whereas longer exposure to the enzymes results in elastolysis.

Staining of elastic tissue before exposure to the enzyme inhibits the elastolytic effect.

We wish to thank the members of the Biochemistry Department of this Institute for advice and assistance.

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NEW ZONE PHENOMENA IN COMPLEMENT-FIXATION REACTIONS.

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ZONE phenomena in complement-fixation reactions have been encountered since the discovery of complement-fixation by Bordet and Gengou (1901). Both pro- and post-zones can occur. A pro-zone in a complement-fixation reaction may be defined as a series of dilutions of immune serum in which complement is fixed incompletely or not at all with appropriate antigen despite good fixation with a series of higher dilutions of the same serum. Such pro-zones have been attributed to antibody excess, haemolysin excess or complement excess. A post-zone results in diminution of the apparent serum titre due to antigen excess. Pro-zones have been observed by Eaton (1941), and Nigg, Crowley and Wilson (1941), post-zones have been described by several workers (Hoyle and Fairbrother, 1937; Lush and Burnet, 1937; Nigg *et al.*, 1941; Henle, Henle, Groupe and Chambers, 1944; Hoyle, 1945). The novel pro-zones described below were encountered in two pools of sera while studying the antigenic relationships of influenza viruses using sera produced in guinea-pigs. They were the only batches of serum out of many so obtained to show such zones.

MATERIALS AND METHODS

Antigens.

The strains used were: Influenza A PR8 and Shope 15; influenza A-Prime A/Eng/47 (Bar) (Barratt); influenza B Lee.

Preparation of purified suspensions of elementary bodies (E.B.).

Francis and Salk's (1942) method of adsorption to and elution from adult fowl red cells was adopted for the purification of the virus elementary bodies. The eggs were chilled at 4° for 3 hr. before harvesting to prevent bleeding. The diluent used was veronal buffer based on the data of Mayer, Osler, Bier and Heidelberger (1946). The purified preparation of E.B. was stored in 1 ml. ampoules at — 76°. The H.A. titre ranged between 1/1280 and 1/10240 (pattern test (Salk, 1944)).

Preparation of soluble antigen (S.A.).

The infected chorioallantoic membranes were removed from the eggs, washed in sterile veronal buffer, and a 20 per cent w/v suspension was made in veronal buffer by grinding with sterile glass powder. The suspension was clarified by centrifugation at 1500 *g* for 20 min. The clear supernatant was adsorbed with 2·5 per cent by volume of washed packed chick cells for 1 hr. twice and centrifuged at 15,000 r.p.m. for 1 hr. in an Ecco centrifuge. The supernatant was clear, had an haemagglutinin titre of less than 1/2 and was stored at 4°. Preparations were discarded after 8 weeks' storage.

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Immune sera.

Eight to 12 weeks old guinea-pigs were anaesthetised by a mixture of chloroform and ether (10 : 90) and 0·5 ml. of a purified suspension of E.B. (H.A. titre 1/1280–1/2560) was instilled into the nose. A single dose was given and the animals were bled after two weeks. The sera were allowed to separate overnight at 4°. The clear blood-free sera were pooled and stored at − 76°.

Complement.

Pooled guinea-pig serum stored in 1 ml. ampoules at − 76° was used.

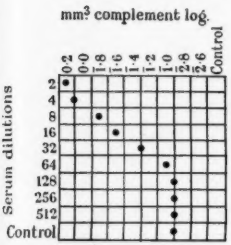


Fig.1

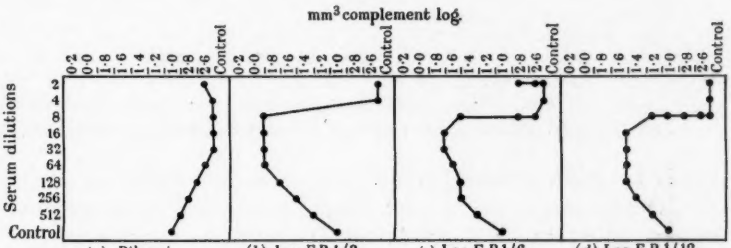
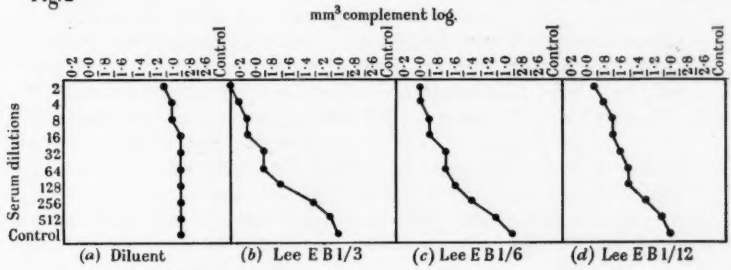


Fig.2

Fig.3



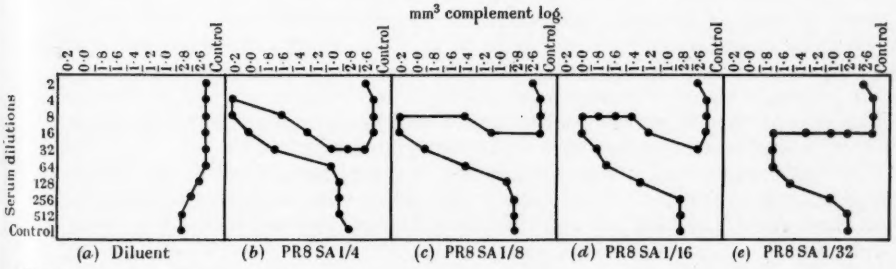


Fig.4

FIG. 1.—Chart showing the recording of a normal pattern of fixation between an immune serum and its homologous antigen.

FIG. 2.—Complement-fixation between Lee E.B. serum 17.v.52 unextracted + Lee E.B. antigen (H.A. titre 1/2560).

FIG. 3.—Complement-fixation between Lee E.B. serum 17.v.52 ether extracted + Lee E.B. antigen (H.A. titre 1/2560).

FIG. 4.—Complement-fixation between PR8 E.B. serum 6.vi.52 unabsorbed + PR8 S.A.

The complement-fixation test.

The tests were performed on perspex sheets as described by Fulton and Dumbell (1949) and by Le Bouvier (1953). Transmitted light was used to read the results because with some of the soluble antigens clear cut " buttons " of unhaemolysed cells could not be obtained. The plate was illuminated from below by oblique light in such a way that the light alone was visible but not the source of the light. A black " Erinoid " plate was used as a background (Ananthanarayan, 1953). This technique permitted easy reading of the results even when the unhaemolysed cells in the drops remained dispersed, and 25, 50 and 75 per cent end-points could be easily estimated though only 50 per cent end-points were recorded.

Results were recorded on charts as shown in Fig. 1. Where the 50 per cent end-point fell between two dilutions the geometric mean was used.

RESULTS.

In the two years of study although most sera fixed complement normally, two pools of sera showed unusual pro-zones and they are the subjects of the study.

1. Lee E.B. serum 17.v.52.

This serum was a pool from 6 guinea-pigs each infected intranasally with 0·5 ml. of Lee E.B. (H.A. titre 1/2560). It did not fix any complement with Lee E.B. antigen diluted 1/3 at serum concentration 1/2 and 1/4 (Fig. 2*b*), but fixation was normal from 1/8 dilution of serum to the end-point at 1/512. The same serum when titrated with Lee S.A. showed no pro-zone but poor fixation, although a sample of Lee convalescent human serum obtained from Dr. A. Isaacs of the World Influenza Centre gave good fixations with the Lee S.A. preparation.

The Lee guinea-pig serum was slightly milky, unlike the usual clear guinea-pig sera. This suggested that a lipoid in the serum might be interfering with fixations in the lower dilutions, while it was diluted out at higher dilutions. The serum was therefore extracted with ether.

Equal quantities of the serum and anaesthetic ether were mixed, well shaken, and left in a narrow separating funnel for 1 hr. at 4°. The serum layer was then drawn off without disturbing or including the ether-serum interfacial layer. The milkiness showed no apparent decrease. The dissolved ether was removed from the serum by exposing it to reduced pressure at laboratory temperature for 15–30 min. The serum was then diluted 1/2 in buffer and inactivated by heating at 56° for 30 min.

This ether-extracted serum was titrated with different antigen concentrations. A control set with unextracted serum was set up in parallel.

With the unextracted serum, the amount of complement fixed in the control without antigen in the lower dilutions was very small, whereas the ether-extracted serum showed the usual anti-complementary pattern of normally behaving serum (Fig. 2*a*, 3*a*).

With Lee E.B., the ether-extracted serum showed no pro-zone such as was still given by the unextracted serum (Fig. 2*b*, *c*, *d* and 3*b*, *c*, *d*).

With Lee S.A. the amount of fixation was markedly increased and the areas were double those obtained with unextracted sera.

2. *PR8 E.B. serum* 6.vi.52.

The second unusual pool came from 6 guinea-pigs, each inoculated intranasally with 0·5 ml. of PR8 E.B. (H.A. titre 1/2560). In an antigen titration

with PR8 E.B. and S.A. against the usual dilution of 1/2 serum, no fixation was observed. A three-dimensional test (Le Bouvier, 1953) using PR8 S.A. was set up to decide whether lack of fixation was due to a pro-zone. The zones of fixation were very peculiar and are believed not to have been described before. They are best appreciated by reference to Fig. 4 (*a–e*). The zone of fixation is marked in the figure by joining the 50 per cent end-points with a line : there is a zone in the region of antibody excess, as well as a zone of no fixation in the intermediate dilutions of complement with 1/4, 1/8 and 1/16 dilutions of serum. Immune sera from rabbits, absorbed with sheep cells to remove heterophile antibodies, usually gave sharp end-points. It was therefore conjectured that the peculiar phenomenon observed with the PR8 guinea-pig serum might be due to the presence of a lysin for sheep cells. The PR8 serum was diluted 1/2 with buffer and adsorbed with 5 per cent washed packed sheep cells for 15 min. at 4°. The three-dimensional test was repeated using S.A. antigen dilutions 1/2–1/32 and serum dilutions 1/2–1/512. A normal pattern of fixation was obtained.

An antigen titration with PR8 E.B. using sheep-cell-absorbed serum gave the normal pattern of fixation.

The comparison of the two abnormal sera.

An attempt was made to correlate the behaviour of the two sera by a further set of experiments. Absorption of the Lee E.B. serum with sheep cells did not enhance the fixation with Lee S.A. in three-dimensional tests. By the time the test against Lee E.B. was performed, the serum had been frozen and thawed several times ; the pro-zone had disappeared.

The PR8 serum was ether-extracted and titrated with PR8 S.A. Ether extraction completely abolished the zones. Repeated freezing and thawing and prolonged storage had not affected the unusual behaviour of PR8 E.B. serum.

Since these unusual zones were seen only in two isolated batches of serum in the course of two years' study, the effects of ether extraction and sheep cell adsorption on normally behaving sera were investigated. Twelve guinea-pigs were inoculated intranasally with PR8 E.B. (H.A. titre 1/2560) ; after two weeks the sera were collected and stored individually. Three of the PR8 sera were selected at random and the areas of fixation with the standard concentration of S.A. were determined with both extracted and unextracted sera. The standard concentration of S.A. is defined as that concentration which fixes 0·5 $mm.^3$ of complement with excess homologous antiserum. Ether treatment neither enhanced nor diminished significantly the areas of fixation of these normally behaving sera.

An immune serum to Shope 15 E.B. from guinea-pigs was titrated with Shope 15 S.A., before as well as after absorption with sheep cells. There was no difference in the areas of fixation. Therefore absorption with sheep cells does not enhance the areas of fixation of normally behaving sera with their homologous antigens.

A complement-fixation test was set up with the unheated and unabsorbed abnormal PR8 serum 6.vi.52, 1/2 dilution, and unsensitised sheep cells and diluent in place of complement. Lysis was observed. This showed that a lysin to sheep cells was present in the unheated unabsorbed serum. The test was repeated using heated unabsorbed serum, complement and unsensitised cells. No lysis

occurred. Therefore, the lysin present was thermolabile. Therefore the phenomena were not due to the active haemolysin since all the sera were heated before use. The interfering substance removed by sheep-cell absorption was either the heat-inactivated haemolysin or some quite different substance.

Discussion.

The Lee E.B. serum had a pro-zone with Lee E.B. This zone could be abolished by ether extraction as well as by prolonged storage and repeated freezing and thawing. The area of fixation of the Lee E.B. serum with Lee S.A. was doubled as a result of ether treatment ; but prolonged storage and repeated freezing and thawing did not enhance the fixation, nor did sheep-cell absorption have any effect on the area of fixation with Lee S.A.

The PR8 E.B. serum had a bizarre zone, one in the region of antibody excess and a second in some intermediate dilutions of complement with some dilutions of serum. This double zone could be abolished by sheep-cell absorption as well as by ether extraction. Repeated freezing and thawing and prolonged storage had no effect on it.

Zones in antibody excess region have been often observed in complement-fixation. Dean (1911) suggested that the zone phenomenon in complement-fixation is analogous to similar phenomena in precipitation and agglutination. He excluded normal haemolysin as an explanation of the phenomenon. In agglutination and lytic reactions zones in antibody excess are common and have been attributed to a specific globulin which acts as an anti-antibody preventing union in higher concentrations of serum but becoming ineffective on dilution (Coca and Kelley, 1921 ; Yanagihashi, 1928 ; Jones and Orcutt, 1934 ; Shibley, 1929 ; Mudd and Mudd, 1927 ; Ward, 1930). The studies of Horsfall and Goodner (1935) and Hartley (1925) on ether extraction of immune sera revealed that " In general, ether extraction reduced the agglutinating and precipitating powers of sera, while combining and protective powers were left unaffected " (Marrack, 1938). It has to be emphasised that the extraction method adopted in the work reported here was neither as vigorous nor as thorough as those adopted by Hartley, and by Horsfall and Goodner ; also these authors were obviously studying normally behaving sera. The only conclusion that can be drawn is that an inhibitor to complement-fixation was present in the sera in low concentration. The inhibitor could be diluted to the point of ineffectiveness or destroyed by ether treatment in the Lee serum and removed by ether treatment or sheep-cell absorption from the PR8 serum. The inhibitors are not the same in the two sera since repeated freezing and thawing did not destroy the inhibitor in the PR8 serum. Repeated freezing and thawing and prolonged storage have been mentioned as causes contributing to the disappearance of zones in sera (Coca and Kelley, 1921).

Summary

Pro-zones in influenza complement-fixation reactions are discussed. Two exceptional batches of convalescent guinea-pig sera showing such zones are described. One serum had a marked pro-zone which was destroyed by ether treatment as well as by prolonged storage and repeated freezing and thawing. The other also exhibited a zone of no fixation in middle dilutions of complement with higher concentrations of sera. This zone was removable by ether

treatment or by sheep cell absorption but did not disappear on storage or after repeated freezing and thawing.

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STUDIES ON THE NATURE OF SILICOSIS : THE POLYMERIZATION OF SILICIC ACID SOLS *IN VIVO*.

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EVIDENCE has been presented (Holt and Osborne, 1953) which suggests that the rôle of silicic acid in silicotic tissue is analogous to that of the muco-polysaccharides of high molecular weight which are present in normal fibrous tissue, and that only highly polymerized silicic acids are capable of producing fibrosis. Orthosilicic acid is formed at the surface of quartz particles at a low pH and, when neutralized by the tissues, the silicate ion must pass through the region of pH 5·5–6 in which polymerization is most rapid. Cement dust also dissolves to produce a high concentration of silicic acid (Holt, 1950), but at an alkaline pH, so that in diffusing away from the dust particle the silicate ion is brought to the pH of the plasma without passing through the pH range in which rapid polymerization occurs. Consequently, in the case of cement, large polymers (polysilicic acid) are not formed and this may explain the relatively harmless nature of cement dust.

We describe here experiments designed to study the factors which affect the polymerization of silicic acid sols *in vivo*. When silicic acid is injected intraperitoneally into rats, it is absorbed and is distributed over the tissues generally, but silicic acids of low molecular weight (oligosilicic acid) also pass readily through the walls of the kidney glomeruli into the urine. The concentration of the glomerular filtrate by the reabsorption of water in the tubules then results in a high silicon level in the kidney, a level which may be some ten or twelve times that of any other tissue (Holt and Yates, 1953). The glomeruli act as ultrafilters, however, and if the silicic acid exists only as polysilicic acid, it is retained in the tissues. The kidney silicon value is then comparable to the general tissue silicon value. A comparison between the kidney silicon value and that of other tissues (*e.g.*, liver) after the injection of silicic acid indicates the degree of polymerization of the tissue silicic acid. Studies have been made using sols at several pH values.

The administration of silicic acid by intravenous injection is not a satisfactory procedure (King, Stantial and Dolan, 1933), since it often results in the death of the animal. Animals invariably survive after the intraperitoneal injection of silicic acid, and oligosilicic acid produces a rapid increase in tissue silicon generally.

METHODS.

The chemical estimation of tissue silicon proved unreliable because the large amounts of phosphorus which are always present interfere. Radioactive ^{31}Si has therefore been used so that its concentration can be measured by physical methods. Its half-life is short (170 min.) necessitating a rapid experimental technique. The activated sample of powdered fused silica was received within 45 min. of its removal from the Harwell pile (BEPO) and the solutions were prepared and injected within a further 30 min.

Solutions were prepared for injection by fusing the irradiated material with 3 times its weight of sodium carbonate and dissolving the melt in water. This solution was then divided into four parts, to each of which dilute HCl was added to bring the pH to 12, 10, 7 and 5·5 respectively. The volume of each part was then adjusted by the addition of distilled water to give a final concentration of 1 mg. SiO_2/ml. Intraperitoneal injections of each solution were immediately made into several rats in the quantities shown in the subsequent Figures.

The animals were killed at predetermined times after injection, then the kidneys and livers were removed and cleared of any adhering tissue. The tissues were rapidly washed with water to remove unabsorbed radioactive material contaminating the surface, and then weighed. After drying in an oven at 100° for 30 min., the tissues were ashed in a platinum crucible and fused with sodium carbonate. Solutions of the melts were then assayed with an annular-type Geiger counter (Veall, 1948). To allow for the decay of the ^{31}Si, the proportion of injected sodium silicate contained in the extracted solutions was determined by a direct comparison with measurements on known dilutions of the solutions used for injection.

RESULTS.

Variation in the Liver and the Kidney Silicon Values with the Time After Injection.

Nine rats were each injected intraperitoneally with 8 ml. of radioactive sodium silicate solution (1 mg. SiO_2/ml.) at a pH of 12. They were killed after 1, 3, 5, 7, 10 and 13hr., single rats being taken up to the 7th hr., and thereafter pairs so that their tissues could be pooled to compensate for the decreased radioactivity of the silicon. The kidneys and livers were removed and treated as previously described to provide solutions for analysis. Two similar experiments of shorter duration were carried out.

After 1 hr. the labelled silicon in the liver was about twice the normal, showing rapid absorption from the peritoneal cavity. After increasing time intervals the value declined slowly until it approximated to that for normal liver silicon (Fig. 1). The radioactive silicon content of the kidney showed a rapid initial rise to a peak value which was some 8 times the normal silicon value after 3 hr.; an equally sharp fall followed to a nearly constant level more than 3 times greater than that found for liver. This level was maintained 13 hr. after the injection. As the maximum difference between the liver and the kidney silicon values occurred 3 hr. after the injection, subsequent comparisons were all made after this time interval.

Variation in Liver and Kidney Silicon Values with Alterations in the pH of the Injected Sol.

Rats were injected with the stated volumes of the silicic acid or silicate solutions at each of the pH values 12, 10, 7 and 5·5. The rats were all killed after 3 hr. and the liver and kidney tissues were assayed.

Solution injected at pH 12 and pH 10.—The liver silicon value increased with increasing dose of the solution at pH 12, giving a maximum value of 109 μg. SiO_2/g. tissue for the maximum dose of 12 mg. SiO_2.

The kidney silicon value increased rapidly to a value of nearly 500 μg./g. (*i.e.*, about 10 times the corresponding value for liver silicon) then rapidly decreased to a value which, at the maximum dose, was lower than that of the liver (Fig. 2). Three other experiments confirmed this result.

When a solution was injected at pH 10, similar results were obtained except

that the corresponding liver silicon values were somewhat lower and the maximum value for kidney silicon occurred at a somewhat higher dose (Fig. 3).

The rate of polymerization of a silicic acid sol increases as its concentration increases and, for a given concentration, is greatest at pH 5·5–6. A sol at pH 10

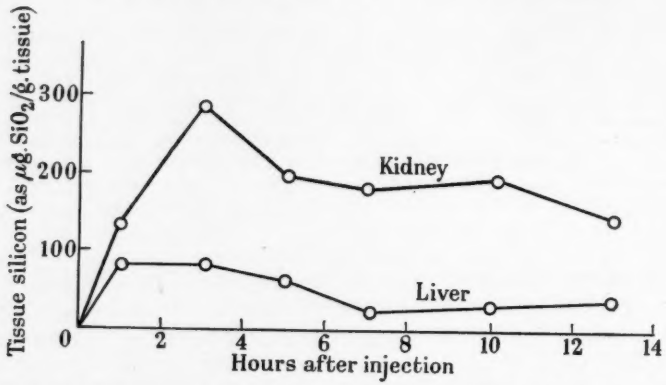

FIG. 1.—Liver and kidney silicon of rats after injecting sodium silicate. Variation with time after injection.
Ordinate : tissue silicon (as μg. SiO_2/g. tissue).
Abscissa : hours after injection.

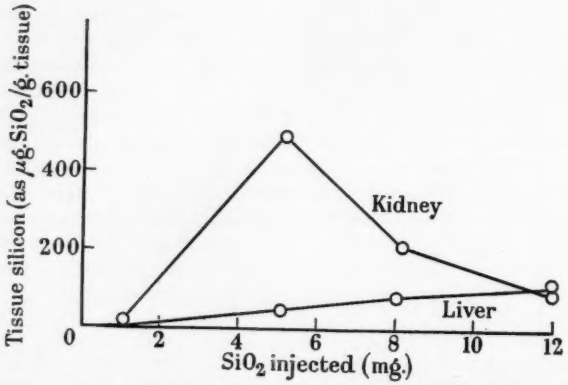

FIG. 2.—Liver and kidney silicon of a rat after injecting sodium silicate brought to pH 12. Variation with amount injected.
Ordinate : tissue silicon (as μg. SiO_2/g. tissue).
Abscissa : SiO_2 injected (mg.).

or 12 contains mainly ions of low molecular weight. On injection, the pH of the sol changes towards 7·4 but at no time is the value in the region in which rapid polymerization occurs. The small polymers pass readily into the blood, the general tissue concentration of the silicon as measured by that of the liver being proportional to the amount injected.

Polymerization at body pH is slow, and at the lower concentrations much of the silicic acid passes through the kidney glomeruli and is concentrated in the tubules. The kidney silicon values are therefore greater than but proportional to the liver silicon values.

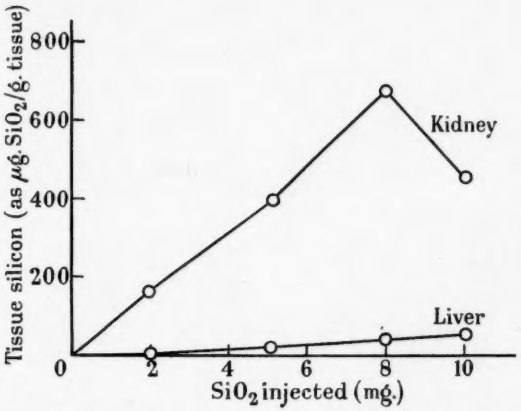

FIG. 3.—Liver and kidney silicon of a rat after injecting sodium silicate brought to pH 10. Variation with amount injected.
Ordinate : tissue silicon (as μg. SiO_2/g. tissue).
Abscissa : SiO_2 injected (mg.)

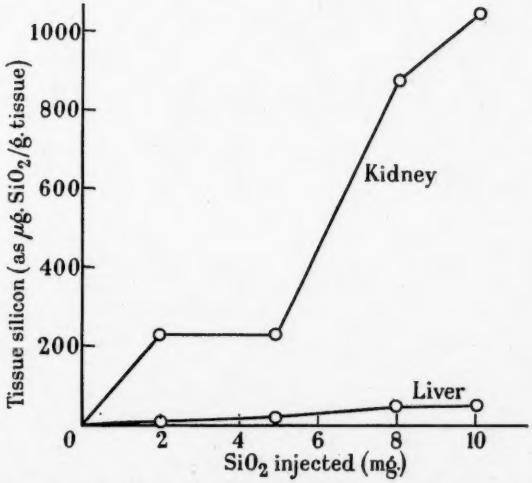

FIG. 4.—Liver and kidney silicon of a rat after injecting sodium silicate brought to pH 7. Variation with amount injected.
Ordinate : tissue silicon (as μg. SiO_2/g. tissue).
Abscissa : SiO_2 injected (mg.).

As the tissue silicon concentration increases, the polymerization rate also increases and polysilicic acid is formed at the expense of the oligo-acid. When the concentration in the liver reaches a value greater than about 50 μg. SiO_2/g. tissue, much of the silicic acid is unable to pass through the walls of the glomeruli and the kidney silicon level falls.

Solution injected at pH 7.—The liver silicon values are somewhat lower than in the previous experiments and the kidney silicon showed no maximum value, continuing to increase with increasing dose. The kidney silicon was over 20 times greater than the liver silicon at the maximum dose (Fig. 4).

Injected at a pH slightly lower than that of the tissues, silicic acid polymerizes more rapidly than do alkaline sols. Its rate of absorption from the peritoneal cavity is therefore slower and the liver silicon level is lower than when the same

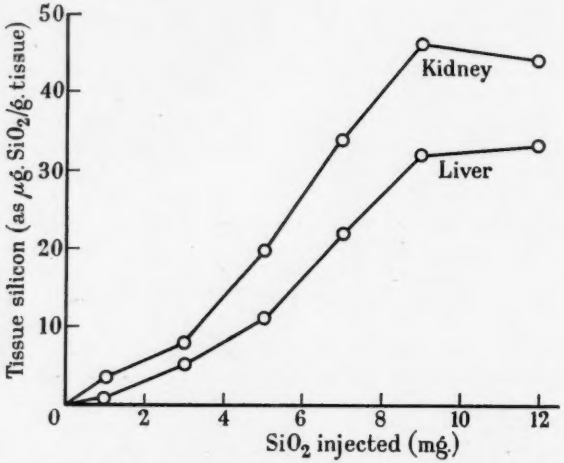

FIG. 5.—Liver and kidney silicon of a rat after injecting sodium silicate brought to pH 5·5. Variation with amount injected.
Ordinate: tissue silicon (as μg. SiO_2/g. tissue).
Abscissa: SiO_2 injected (mg.).

silicon dose is administered at a higher pH. Under these conditions the tissue concentration never reaches a value such that increased polymerization causes a decreased kidney silicon value.

Solution injected at pH 5·5.—The liver silicon values were lower than in the previous experiments and the kidney silicon values were almost identical with those of the liver (Fig. 5).

At the pH of maximum polymerization, polysilicic acid is rapidly formed and is absorbed very slowly from the peritoneum. The polymers which do reach the blood may travel via the lymphatics. They are too large to pass through the walls of the glomeruli. Consequently, there is no concentration in the kidney and the level of the kidney silicon becomes identical with that of the tissues generally and of the liver in particular.

DISCUSSION.

The theory that the abnormal fibrous tissue in silicosis is produced by silicic acid which results from the solvent action of the tissue fluids on the dust particles is now firmly established. Evidence has been reviewed by King (1947), who concluded that solubility measurements support the hypothesis, the amount of silicic acid passing into solution from most pathogenic dusts, such as quartz and flint, being greater than that from the least pathogenic dusts, such as shale.

King was careful to emphasize some anomalies, however, which are not easily explained by the simple solubility theory. This was earlier emphasized by the Committee on Industrial Pulmonary Diseases of the Medical Research Council (Report, 1945): "Although it may be true in general that the more soluble a siliceous dust, the greater its toxicity, many variations from this generalization are shown in the South Wales mine dusts." Gardner (1938) studied the toxicities of one series of dusts and found no correlation between the amount of soluble silica liberated in suspensions of the dusts and the degree of fibrosis they produced when injected. The case of cement, which releases high concentrations of silicic acid in the body (Holt, 1950) but is relatively harmless, has been discussed (Holt and Osborne, 1953) and another necessary factor in the formation of silicotic tissue was deduced, namely, the polymerization of the silicic acid.

The experiments described here illustrate two of the factors which control the polymerization of the silicic acid *in vivo*. These are pH, polymerization being much more rapid in the range 5·5–6, and concentration. Plainly, if at any stage the silicic acid is brought to pH 5·5–6, as happens when quartz particles dissolve in tissue fluids (Holt and Osborne, 1953), polysilicic acid may form, but even at pH 7·4 the formation of polysilicic acid is possible if the silicic acid concentration is high enough.

It is difficult to believe that the concentration of silicic acid formed by the dissolution of scattered dust particles in the lung can reach a value high enough for the formation of polysilicic acid. It is characteristic of silicosis, however, that the dust particles are collected by phagocytes which become heavily laden and migrate to form clumps. Writing of experimental silicosis, Belt, Ferris and King (1940) stated: "Rat lesions begin as a cluster of silica-laden alveolar phagocytes." And again, "The less noxious dusts, producing only the simplest of foreign body reactions, tend to be dispersed into smaller and smaller aggregations, which become more and more widespread." "More harmful dusts on the other hand, tend to retain a nodular distribution, and sometimes coalescence is carried to the point of producing large confluent lesions" (Belt and King, 1945). It is these masses of dust-laden cells, both in the lung and in the lymph glands, which become surrounded by a thick layer of fibrous tissue. In these clumps the number of particles, and consequently the surface area of silica exposed, is very large. A local concentration of silicic acid high enough for the production of polysilicic acid may be built up.

Here also is an explanation of the observation that low concentrations of silica dust do not produce fibrosis. Quartz may be present in non-silicotic lungs (Hicks, McElroy and Warga, 1937). If the particles are isolated, the concentration of silicic acid is nowhere sufficient for the production of high polymers.

The silica condensate which is formed in furnaces where silica is fused has a very small particle size and is highly soluble, but it is harmless (King, 1947). Even if highly soluble, an isolated silica particle is unlikely to produce silicic acid in a

concentration sufficiently high for polymerization to proceed very far. Most particles are ingested by phagocytes within 20 to 30 hours of their arrival in the alveoli (Belt and King, 1945) but the migration of phagocytes is a slow process (Haythorn, 1913 ; Permar, 1920), and the dissolution of these readily soluble particles is probably completed long before agglomerates of silica-laden particles can form. Consequently, little polymerization of the silicic acid can occur.

Confirming this conception are the results of experiments recently described by King, Mohanty, Harrison and Nagelschmidt (1953). They injected graded flint dusts of five different size ranges intratracheally into rats, the doses being adjusted so that the same surface area of dust was administered to every animal. The dust of size range 1–2 μ produced the maximum fibrosis in the shortest time. Finer or coarser particles were less pathogenic. The low pathogenicity of the finest (0·5 μ) fraction is puzzling when considered in the light of the simple solubility theory, and the authors state that " there was no difference in the ease of phagocytosis as far as could be judged by the sections and no other obvious explanation can be seen."

It was earlier argued that isolated silica particles in the lung are unlikely to produce polysilicic acid. Two factors will then operate antagonistically. On reaching the alveoli, a silica particle will begin to dissolve. The silicic acid formed from the isolated particle polymerizes only to a small extent, it diffuses rapidly and is detoxicated (Holt and Yates, 1953) or is eventually excreted (King and McGeorge, 1938). At this stage, therefore, the degree of elimination of the silica particle is inversely proportional to its particle size.

Once the particles are agglomerated into patches by phagocytosis, the amount of silica surface exposed is locally very large, and a concentration may be built up which will allow considerable polymerization and, consequently, fibrogenesis. The tissue damage is now greater as the rate of solution is greater, and now a given mass of smaller particles is more effective than the same weight of larger particles. Since these two effects are opposed, an optimum particle size is to be expected.

Many observations suggest that inert dusts have a neutralizing effect on the pathological action of silica (Mavrogordato, 1918 ; Gossner, 1936 ; Naesland, 1940). The evidence is not conclusive but so many examples have been reported that it cannot be dismissed. The action of the inert dust has been ascribed to a reduction in the amount of silicic acid released by the silica particles although some of these dusts have but little effect on the dissolution of silica.

If a mixture of silica and other dust particles is ingested by a phagocyte, the mixed load is likely to contain fewer silica particles than if only silica particles are ingested. Consequently, in the agglomerates of phagocytes, the total surface presented by the silica particles in a unit volume of tissue is likely to be less when a mixed dust is inhaled even though the total amount of silica in the lung is the same in either case. Polymerization and the development of fibrous tissue will be more advanced in the areas of high silica concentration. But if there is a high enough silica concentration after mixed dust inhalation, the fibrosis may well be more extensive. The effect cannot be regarded as a simple one, however, since the inert dust may act chemically either to accelerate or to retard the polymerization of the silicic acid produced by the silica particles.

Examined in the light of a theory which regards both the solubility of the dust particles and the subsequent polymerization of the solute as essential factors in

the production of silicotic tissue, the suggestion made by Mavrogordato (1918), Briscoe, Holt, Matthews and Sanderson (1937) and others, that inert dusts may be useful in combating silica dusts, has a new significance. Possible explanations for the anomalous results which have been obtained in previous investigations are apparent and point to the need for extended research.

SUMMARY.

Two factors in the polymerization of silicic acid *in vivo*, concentration and pH, have been studied by injecting ^{31}Si-labelled silicic acid into rats and comparing the ^{31}Si contents of the liver and kidney tissues.

A sol at pH 5·5 polymerizes rapidly and is absorbed slowly. The silicic acid which reaches the tissues generally is too highly polymerized to pass through the kidney glomeruli.

At pH 7 polymerization is slower. Absorption occurs but the tissue concentration is never so high that large polymers form.

Injected at pH 10 and 12, little polymerization occurs in the peritoneal cavity. Absorption is rapid and, when the tissue concentration exceeds a certain value, large polymers are formed.

The importance of the polymerization of silicic acid in the formation of fibrous tissue is discussed. The discussion is extended to explain some apparent anomalies in the application of the solubility theory of silicosis to certain dusts.

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THE PRODUCTION OF INCOMPLETE VIRUS PARTICLES AMONG INFLUENZA STRAINS: EXPERIMENTS IN EGGS

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CONCENTRATED allantoic inocula of the classical influenza strain PR8 give rise to the production of incomplete virus particles possessing the same antigenic and enzymic equipment as the parent virus but lacking infectivity (von Magnus, 1947). The first exception to this phenomenon was noticed incidentally in the course of some unrelated work. The observation, however, seemed interesting enough in its implications to warrant a systematic comparison of influenza strains in this regard. From the results we hoped to gain information on two specific points: first, the relationship between the von Magnus phenomenon and interference; and second, the nature of the difference between the initial and subsequent cycles of viral multiplication, a fact which has been explicitly recognized only recently (Cairns, 1952; Fazekas de St.Groth and Cairns, 1952).

A preliminary note (Fazekas de St.Groth and Graham, 1953), restricted to the behaviour of two contrasting strains, has appeared. Here the detailed findings with ten influenza virus strains are presented, and it will be shown that the von Magnus phenomenon, far from being a uniform feature of the strains tested, shows such quantitative variation as to give the impression of fundamental differences between the viruses at the opposite ends of the series.

MATERIALS AND METHODS.

Saline was made up as 0·90 per cent NaCl in distilled water. The diluent used in infectivity titrations was a mixture of 1 part Seitz-filtered horse serum and 9 parts sterile saline.

Red blood cells were obtained from venous blood of White Leghorn fowls, collected in a 2 per cent sodium citrate solution. They were washed in three changes of saline, packed and stored at 4°. Suspensions of 10 per cent strength (by volume) were made up daily. No cells older than three days were used.

Viruses used in this study were grown by the standard method laid down by Beveridge and Burnet (1946). Batches of six 11-day-old eggs were inoculated allantoically with 0·1 ml. sterile horse serum saline containing not more than 10^4 infective units of virus. After 42 hr. incubation at 35° the eggs were chilled for 2 hr. and the allantoic fluids harvested aseptically. The virus preparations were always used within 3 hr. of harvesting.

Type A strains:

- WSE sub-strain of WS, the prototype of influenza A virus (Smith, Andrewes and Laidlaw, 1933), adapted by extensive serial passage to grow and produce pocks on the chorio-allantois (Burnet, 1936);
- PR8 (Francis, 1934);
- MEL (Burnet, 1935);
- BEL (Burnet, Beveridge, Bull and Clark, 1942).

Type A-prime strains :
CAM (Anderson, 1947) ;
FM1 prototype of A-prime virus (Rasmussen, Stokes and Smadel, 1948).

Type B strains :
LEE prototype of influenza B virus (Francis, 1940) ;
BON (Beveridge, Burnet and Williams, 1944) ;
HUT (Burnet, Stone and Anderson, 1946).

Swine influenza :
SW the classical " Strain 15 " of Shope (1931).

Haemagglutinin titration.—Serial twofold dilutions of the test material were made in 0·25 ml. volumes of saline. To each tube a standard drop (0·025 ml.) of a 10 per cent fowl red cell suspension was added, the racks shaken and left standing at room temperature (18–24°). The pattern of the settled cells was read 20 min. later, a conventional degree of partial agglutination marking the end-point. The titre of a sample is the reciprocal of this end-point dilution. In Table I haemagglutinating units (HA) refer to 1·0 ml. volumes, *i.e.*, the actual titres have been multiplied by a factor of 4. As the error of haemagglutinin titrations shows a log-normal distribution, all titres have been conveniently expressed in $\log_{10}$ units.

Infectivity titration.—Serial 3·16-fold (log 0·5) dilutions of the test material were prepared in cold horse serum saline, and all tubes were kept in an ice-bath until used. Sufficient penicillin was added to the diluent to give a concentration of 100 units per egg. Of the dilutions 0·10 ml. volumes were inoculated allantoically into groups of five 11-day-old embryos. The eggs were incubated at 35° for 45 hr.

Then, without preliminary chilling of the eggs, a hole 3 mm. diam. was poked through the paraffin seal with a red-hot iron rod, and through it a sample of approximately 0·2 ml. of allantoic fluid was removed with a special " dipping pipette ". This pipette was made of 7 mm. external diameter glass tubing by pulling it out in a flame, sealing the tip, and blowing a small hole through the side of the drawn-out part approximately 4 mm. from the tip. This device is a small version of the " harvesting pipette " designed by Henle and Henle (1944) ; with its aid samples of allantoic fluid may be conveniently collected without any interference from extra-embryonic structures, which render sampling with ordinary Pasteur pipettes cumbersome. The dipping pipettes were sterilized in boiling water between each egg.

One standard drop of 10 per cent red cells was added to each sample of allantoic fluid, and the pattern of agglutination read after 20 min. As the reactions were invariably either complete agglutination or complete lack of agglutination, the results were recorded as positive or negative. The cumulative totals of positive (infected) and negative (non-infected) eggs were calculated, and the modal infectivity end-point determined by the method of Reed and Muench (1938). As with haemagglutinating units (HA), the infectivity titres were corrected to refer to 1·0 ml. volumes of allantoic fluid, and are expressed in $\log_{10}$ units. In Table I they appear under the symbol "ID".

EXPERIMENTS.

Single-passage Tests for Production of Incomplete Virus.

The first set of experiments consisted essentially in the replication, with each strain to be tested, of the fundamental experiment described by von Magnus, namely the titration of infective and haemagglutinating units after allantoic inoculation of large or small quantities of influenza virus. To avoid any bias in favour of incomplete virus production, the test samples were always taken at the height of infectivity, as assessed in some preliminary trials. There is no need to give here the results of these sighting experiments in detail as they were in complete agreement with previously published findings of other workers. It will suffice to say that after inoculation of concentrated virus preparations (10^9–10^{10}

ID_{50}) the allantoic fluids were harvested after 16 hr. incubation; after dilute inocula (10^4–10^5 ID_{50}), where three or more cycles of multiplication are needed to reach maximum titres, the fluids were harvested after 42 hr.

All experiments were done to the following set pattern. Two days before the main tests the strain to be used was given a single allantoic passage at high dilution, usually 10^{-4}. By this means it could be ensured that the seed would contain no incomplete virus. The fluid obtained was titrated for haemagglutinin, and if found suitable, *i.e.*, of high titre, it was used within 3 hr. of harvesting. The experiment proper was done on two groups of six 11-day eggs. One group received 0·6 ml. of the undiluted seed virus allantoically, and was harvested after 16 hr. at 35°. The other group was given 0·05 ml. of a 10^{-4} dilution of the same seed in horse serum saline; the allantoic fluid of these eggs was removed after 42 hr. incubation. Immediately after harvesting approximately 1 ml. of each allantoic fluid was ampouled and stored in the frozen state at −20°. On the rest of the allantoic fluids haemagglutinin tests were performed on the day of harvesting.

TABLE I.—*Yield following Infection*

Strain.	Inoculum: ~ 10^9 ID_{50}. HA/ml.	ID_{50}/ml.	ID/HA ratio.	Inoculum: ~ 10^4 ID_{50}. HA/ml.	ID_{50}/ml.	ID/HA ratio.
Type A, WSE	3·40	6·75	3·35	3·97	9·25	5·28
	3·22	7·42	4·20	3·58	10·20	6·72
	3·43	7·46	4·03	3·67	10·40	6·73
	3·09	7·41	4·32	3·67	9·70	6·03
	3·10	7·75	4·65	3·58	10·20	6·62
	2·20	6·89	4·69			
	2·17	6·75	4·58	2·98	9·12	6·14
	Average ratio: 4·26 ± 0·18			Average ratio: 6·25 ± 0·23		
„ PR8	3·45	7·14	3·69	3·92	10·22	6·30
	3·30	7·72	4·42	3·44	10·34	6·90
	3·14	7·30	4·16	3·51	9·42	5·91
	3·42	7·55	4·13	3·22	10·30	7·08
	3·51	7·24	3·73	3·47	9·39	5·92
	3·22	7·70	4·48	3·30	10·19	6·89
	Average ratio: 4·10 ± 0·14			Average ratio: 6·50 ± 0·22		
„ MEL	3·19	7·80	4·61	3·40	10·10	6·70
	3·16	8·28	5·12	3·55	9·36	5·81
	3·31	7·50	4·19	2·53	8·75	6·22
	3·28	7·00	3·72	3·11	10·00	6·89
	3·25	7·75	4·50	3·41	10·00	6·59
	3·28	7·75	4·47	3·32	10·00	6·68
	3·28	7·25	3·97	3·53	9·40	5·87
	Average ratio: 4·37 ± 0·17			Average ratio: 6·39 ± 0·16		
„ BEL	4·03	8·90	4·87	4·05	9·88	5·83
	3·85	9·35	5·50	4·05	9·75	5·70
	3·52	8·33	4·81	3·23	9·80	6·57
	3·37	8·38	5·01	3·53	10·20	6·67
	3·52	7·43	3·91	3·57	9·65	6·08
	3·55	8·63	5·08	3·83	9·75	5·92
	Average ratio: 4·86 ± 0·21			Average ratio: 6·13 ± 0·16		

Infectivity was titrated later, on the frozen samples. Tests on the two experimental groups were always done in parallel. If any of the titrations proved technically unsatisfactory, there was enough of the frozen sample for several repetitions.

Table I gives the results in detail. All titres are expressed in $\log_{10}$ units and are directly comparable as they refer to 1·0 ml. volumes of allantoic fluid. The infectivity-to-haemagglutinin ratio of individual samples is shown, and the average ratio and its standard error has been calculated for all groups. The Table also contains a test of significance for the comparison of yields after the two inocula. Although only two averages are being compared at a time, the method applied is the analysis of variance instead of the more usual *t*-test. This was done mainly because the statistics so obtained could be used directly in later inter-strain comparisons. It should be clear, of course, that the two methods are bound to give identical results; indeed, Student's *t*-test is but a simpler special case of the more comprehensive analysis of variance.

with Concentrated or Dilute Seed.

Analysis of Variance.

Source of variation.	Sum of squares.	Degrees of freedom.	Variance.
Between inocula	13·4067	1	13·4067
Error	2·9173	11	0·2652
Total	16·3240	12	

Variance ratio F = 50·55; P < 0·001.

Between inocula	17·2559	1	17·2559
Error	1·9295	10	0·1929
Total	19·1853	11	

Variance ratio F = 89·43; P < 0·001.

Between inocula	16·3623	1	16·3623
Error	2·3677	12	0·1973
Total	18·7300	13	

Variance ratio F = 82·93; P < 0·001.

Between inocula	4·8006	1	4·8006
Error	2·1923	10	0·2192
Total	6·9929	11	

Variance ratio F = 21·90; P < 0·001.

[*Table continued overleaf.*

TABLE I—

Strain.	Inoculum : $\sim 10^9$ ID_{50}. HA/ml.	ID_{50}/ml.	ID/HA ratio.	Inoculum : $\sim 10^4$ ID_{50}. HA/ml.	ID_{50}/ml.	ID/HA ratio.
Type A-Prime, CAM	2·95	8·10	5·15	2·83	8·90	6·07
	3·58	9·78	6·20	2·83	9·72	6·89
	3·10	9·25	6·15	3·37	9·06	5·69
	3·34	8·42	5·08	3·58	9·65	6·07
	3·16	9·30	6·14	3·43	9·25	5·82
	3·07	8·00	4·93	2·20	9·15	6·95
	3·28	8·60	5·32	3·37	9·60	6·23
	Average ratio : 5·57 ± 0·22			Average ratio : 6·25 ± 0·19		
„ „ FM1	2·68	7·50	4·82	3·01	10·35	7·34
	2·77	7·85	5·08	2·80	9·07	6·27
	2·95	8·35	5·40	3·40	8·20	4·80
	2·11	7·70	5·59	2·44	8·86	6·42
	3·07	8·88	5·81	2·95	7·90	4·95
	3·19	8·88	5·69	2·56	9·28	6·72
	Average ratio : 5·40 ± 0·16			Average ratio : 6·08 ± 0·41		
Type B, LEE	3·28	9·81	6·53	3·41	9·45	6·04
	3·23	9·20	5·97	3·28	9·80	6·52
	3·23	9·62	6·39	3·33	9·40	6·07
	2·92	9·82	6·90	3·23	9·02	5·79
	3·32	9·63	6·31	2·98	9·80	6·82
	3·23	9·30	6·07	3·10	9·30	6·20
	3·55	10·05	6·50			
	3·19	9·80	6·61	1·00	7·55	6·55
	3·49	10·05	6·56			
	3·37	8·92	5·55	3·48	9·17	5·69
	3·58	9·42	5·84	3·22	9·00	5·78
	3·04	8·63	5·59	1·72	7·86	6·14
	2·95	8·50	5·55	2·02	7·86	5·84
	3·37	9·38	6·01			
	3·07	8·76	5·69	3·04	9·66	6·62
	Average ratio : 6·14 ± 0·04			Average ratio : 6·17 ± 0·03		
„ BON	2·05	6·72	4·67	2·83	8·60	5·77
	2·05	6·35	4·30	2·92	8·06	5·14
	2·35	6·50	4·15	3·10	7·87	4·77
	2·05	6·10	4·05	2·68	8·00	5·32
	2·05	5·65	3·60	2·68	8·50	5·82
	2·92	6·28	3·36	2·74	9·00	6·26
	Average ratio : 4·02 ± 0·19			Average ratio : 5·51 ± 0·22		
„ HUT	2·68	7·42	4·74	2·68	8·93	6·25
	2·41	8·00	5·59	2·80	8·50	5·70
	2·38	7·22	4·84	2·20	7·85	5·65
	2·92	8·62	5·70	2·38	8·78	6·40
	2·27	7·42	5·15	2·65	8·36	5·71
	2·58	8·50	5·92	2·83	9·06	6·23
	Average ratio : 5·32 ± 0·20			Average ratio : 5·99 ± 0·14		
Swine Influenza, SW	3·10	7·43	4·33	3·37	9·20	5·83
	3·13	7·50	4·37	2·83	9·50	6·67
	2·83	7·58	4·75	3·13	8·81	5·68
	2·98	7·20	4·22	2·80	9·28	6·48
	3·28	8·32	5·04	3·41	10·00	6·59
	3·55	8·40	4·85	3·53	9·70	6·17
	Average ratio : 4·59 ± 0·14			Average ratio : 6·24 ± 0·17		

—*continued.*

Analysis of Variance.

Source of variation.	Sum of squares.	Degrees of freedom.	Variance.
Between inocula	1·6114	1	1·6114
Error	3·4102	12	0·2842
Total	5·0215	13	

Variance ratio $F = 5·67$; $0·05 > P > 0·01$.

Between inocula	1·4076	1	1·4076
Error	5·7913	10	0·5791
Total	7·1989	11	

Variance ratio $F = 2·43$; $0·20 > P > 0·05$.

Between inocula	0·0076	1	0·0076
Error	4·2463	25	0·1699
Total	4·2539	26	

Variance ratio $F = 0·05$; $P > 0·5$.

Between inocula	6·6751	1	6·6751
Error	2·5775	10	0·2578
Total	9·2526	11	

Variance ratio $F = 25·90$; $P < 0·001$.

Between inocula	1·1532	1	1·1532
Error	1·9244	10	0·1924
Total	3·0776	11	

Variance ratio $F = 5·99$; $0·05 > P > 0·01$.

Between inocula	8·1013	1	8·1013
Error	1·4004	10	0·1400
Total	9·5017	11	

Variance ratio $F = 57·85$; $P < 0·001$.

Plentiful production of incomplete virus is evident with the strains WSE(A), PR8(A), MEL(A) and SW. The von Magnus phenomenon is as conspicuous in these as it was with the two PR8 sub-strains on which the work of the Scandinavian authors had been performed. The ID/HA ratios dropped from the normal average to values lower by about two orders of magnitude. The BEL(A) and BON(B) strains formed relatively smaller proportions of non-infective particles after inoculation of undiluted seed, although the reduction of the ID/HA ratios is still highly significant. The two A-prime viruses, CAM and FM1, and the HUT(B) strain are borderline cases, the drop in the average ID/HA ratios being, at the most, suggestive but statistically not significant. The LEE(B) strain, on the other hand, did not even give a hint of incomplete virus production. Since this behaviour was in such striking contrast to the strains exhibiting the von Magnus phenomenon, four separate series of tests were made with the LEE strain. The results proved to be reproducible, showing consistently no difference between the yields of virus after concentrated or dilute infective inocula.

Statistical.

The data in Table I thus give the impression of considerable diversity among influenza strains in incomplete virus production. As it seemed desirable to assess the contribution of different variables entering into the experiments, the results were submitted to an analysis of variance. This method is applicable provided the variances of all sub-groups to be compared are homogeneous. However, there was some reason to doubt whether this postulate would be automatically fulfilled, as further sources of variation might have emerged with the production of incomplete virus. Consequently, the variances of all sub-groups were first compared by Bartlett's test. According to the formula

$$\chi^2_{(k-1)} = \frac{k(n-1)\ln \bar{V} - (n-1)\Sigma \ln V}{1 + [(k+1)/3(n-1)k]},$$

where V is the variance of any of k sub-groups containing n replicates, the value of $\chi^2_{(19)} = 17{\cdot}045$. This corresponds to a probability of $P = 0{\cdot}62$, *i.e.*, there is no significant deviation from homogeneity, and an analysis of variance may be set up in its standard form. This result is equivalent to saying that whatever further sources of error have been introduced by the presence of incomplete virus in some of the specimens, their total effect is negligible compared with the inherent inaccuracy of infectivity titrations.

TABLE II.—*Statistical Evaluation of the Results in Table I*

Total Analysis of Variance.

Source of variation.	Sum of squares.	Degrees of freedom.	Variance.	Variance ratio.	Probability.
Inocula	45·5303	1	45·5303	191·19	<0·001
Strains	22·0573	9	2·4508	10·29	<0·001
Interaction	23·4313	9	2·6035	10·93	<0·001
Error	28·5769	120	0·2381		
Total	119·5958	139			

Analysis of Interaction-variance in Respect to Strains.

Strains at 10^3 ID_{50}	41·5475	9	4·6164	22·45	<0·001
Error	12·7477	62	0·2056		
Sub-total	54·2952	71			
Strains at 10^4 ID_{50}	3·9411	9	0·4379	1·60	0·16
Error	15·8292	58	0·2729		
Sub-total	19·7703	67			

The analysis of variance on all data obtained from the single-passage experiments is given in Table II. The important finding is the absence of difference among the strains after dilute infective inocula, as opposed to the highly significant difference in the yields after infection with large amounts of virus. The former confirms the conclusion we reached in an earlier study (Fazekas de St.Groth and Cairns, 1952), namely that the ID/HA ratio is identical for all well-adapted influenza strains when grown in the allantois from dilute seed, and that under these conditions no incomplete virus is being formed. The latter provides statistical evidence that the von Magnus phenomenon cannot be elicited uniformly with all influenza viruses. This point is stressed also by the highly significant interaction between strains and inocula, that is, while in some strains the size of the inoculum makes a difference, in others it does not.

We have then within the group of influenza viruses strains which produce less than 1 per cent viable offspring if inoculated into the allantois in amounts of 10^9 ID_{50}. At the other extreme there are strains in which incomplete virus is not detectable by the technique employed. As these differences comply with the strictest criteria of significance, the production of incomplete virus has to be regarded as a property with wide quantitative variation among influenza viruses. On the basis of the above results a *gradient of incomplete virus production* may be established, giving the series PR8 > WSE, MEL > SW > BON, BEL > HUT, CAM > FM1 > LEE, in the order of decreasing proneness to form non-infective particles.

Serial Passage Tests for Production of Incomplete Virus.

The single-passage experiments have revealed differences among influenza strains in regard to incomplete virus production. Yet, due to the inherent limitations of the technique, it was not possible to settle the question whether these differences were absolute, *i.e.*, whether some of the strains produced no incomplete particles at all. We sought to solve this problem by carrying concentrated inocula through several consecutive passages ; thus the effect can be cumulated while the error of the titrations remains constant. Were no incomplete particles produced, the series should be expected to behave like a serial passage of dilute inocula, *viz.*, maximal infectivity and haemagglutinin titres would be obtained throughout. If, on the other hand, even small amounts of incomplete particles were produced, these would progressively interfere with the yields of virus in subsequent passages, and finally a reduction of both infectivity and haemagglutinin titres should be expected. In experiments of this type special care has to be taken (*a*) to expose the great majority of cells to the initial inoculum, *i.e.*, to work at high multiplicities, and (*b*) to avoid contamination of the first-cycle yield with virus from later cycles which were initiated under different conditions. The latter precaution is particularly important in view of the observation that infection of a larger number of cells acts as a mitogenetic stimulus on the allantoic wall, resulting in the appearance of new infectible cells in numbers sufficient to obscure the results (Fazekas de St.Groth and Graham, 1954).

The technique finally adopted and applied to all strains was as follows : The seed virus was given one allantoic passage at 10^{-4} dilution immediately before the experiment proper. Then three 11-day eggs were inoculated allantoically with 2·0 ml. each of undiluted seed, and incubated for 12 hr. The fluids were then harvested through the hole used for inoculation, without previous chilling of the eggs. Three ml. volumes of each fluid were used to prepare pools within each group, and of these pools again 2·0 ml. volumes were inoculated into each of a group of three eggs, and the procedure repeated through four passages. The original seed, each of the first, second and third passage pools, and all the three

fluids of the final passage were titrated individually for infectivity and haemagglutinin content either immediately or after storage at −20°.

The results with six representative strains are shown in the Figure. The strains were so chosen that the group should contain a virus of each immunological type exhibiting the von Magnus phenomenon (PR8(A), BON(B) and SW), and also three strains which did not produce significant amounts of incomplete virus in the single-passage experiments above : CAM(A′), FM1(A′) and LEE(B). The four strains not shown in the figure behaved in every respect like the viruses PR8, BON and SW.

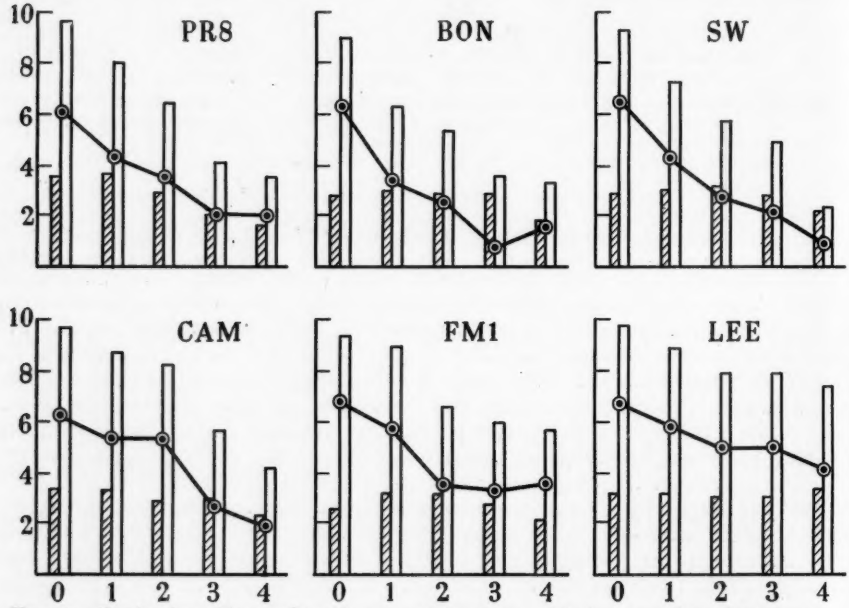

FIGURE.—Production of incomplete virus on serial passage. Abscissa : number of passages. Ordinate : titre in $\log_{10}$ units. Open columns : infectivity titre. Hatched columns : haemagglutinin titre. Continuous line : ratio of infectivity/haemagglutinin. (For experimental details, see text.)

The findings with the strains PR8, BON and SW are a replication of von Magnus' findings : consecutive passage of large inocula progressively reduced the proportion of infective virus in the yields, and the ID/HA ratios are approximately a thousand times lower than the normal ID/HA ratio ($10^{6.26}$) already in the second passage and onwards. It is worth noting that from the stage where the absolute amount of infective virus in the inoculum dropped to a level below 1 per cent of infectible cells, that is to less than 10^6, the haemagglutinin yield of the eggs was also reduced. This phenomenon is well accounted for by the fact that under these conditions a second cycle of multiplication became possible, and in this cycle the interfering effect of the originally inoculated excess of incomplete virus became noticeable. It is known from previous work (Fazekas de St.Groth, Isaacs and Edney, 1952) that the establishment of heterologous inter-

ference is not instantaneous, and thus the first-cycle yields of active virus are the same whether the initial seed contained infective particles only or a mixture of infective and inactivated particles. A numerical estimate of this reduction may be supplied by comparing the mean haemagglutinin titres of the " O " and " I " passages with the average titre of the three fluids harvested after the fourth passage. The reduction for PR8 is 3·62 — 1·59 = 2·03 $\log_{10}$ units, *i.e.*, 100-fold ; for BON 2·83 — 1·78 = 1·05, *i.e.*, 11-fold ; for SW 2·89 — 2·16 = 0·73, *i.e.*, 5-fold. These differences are all significant, the error of a single haemagglutinin titration being $\pm$ 1·3-fold.

Of the two borderline cases, CAM(A′) and FM1(A′), the former obviously belongs to the group of viruses which exhibit the von Magnus phenomenon. In the single-passage experiments the CAM-strain showed a production of incomplete virus which was significant at the 5 per cent but not at the 1 per cent level. This behaviour was maintained through two consecutive passages, but from the third onwards the cumulative effect showed up quite clearly and the ID/HA ratios dropped well below the normal, the difference being over 1000-fold. Also, there is a distinct reduction in haemagglutinin yields with a difference of 3·33 — 2·21 = 1·12, *i.e.*, 13-fold.

The other A-prime strain, FM1, cannot be classed with the previous group of viruses inasmuch as the total reduction of infective offspring never reaches the low levels attained by the strains showing the typical behaviour described by von Magnus. Whereas the first four viruses represented in the figure produce an excess of incomplete particles of the order of 30,000 to 1 by the end of the fourth passage, the FM1-strain reaches a ratio of $<1000:1$ after the second passage and stays at this level for the subsequent passages. There can be no doubt, however, that significant amounts of incomplete virus have been produced and this became detectable by the more rigorous test afforded by the repeated-passage experiments ; there was also a definite drop in haemagglutinin yields, namely 2·88 — 2·14 = 0·74, *i.e.*, 5-fold.

The LEE strain in which no difference could be detected by the single-passage test, could also be shown to produce some incomplete virus under these conditions. The reduction of the ID/HA ratios is, however, evidently less than in any of the other strains tested, being of the order of 100 even after four consecutive passages. It is also significant that, though the multiplicity of infection was kept at the level of 50 particles for each infectible cell throughout the four passages, there is no reduction of haemagglutinin yields within the series. The average haemagglutinin titres compared as in the other strains show that the final yield was 3·06 — 3·23 = —0·17, *i.e.*, unchanged, the minimal rise (1·2-fold) being insignificant.

The general conclusion to be drawn from this set of experiments is that production of incomplete virus can be elicited from every one of the influenza virus strains tested. The differences found are thus, although extreme, of a quantitative nature rather than absolute. From the practical point of view the behaviour of haemagglutinin titres may be of importance. Their trend indicates that by the technique generally used, *viz.*, repeated passaging at high concentrations, no useful further reduction of the ID/HA ratio can be hoped, as below a limiting concentration of infective particles in the seed the resultant haemagglutinin yields will drop. Thus they not only fail to give preparations with a larger proportion of incomplete particles in them, but actually lead to a lower total output of particles per egg.

DISCUSSION.

Tests for incomplete virus are necessarily bound up with the observation of a lowered yield of infective particles, suggesting some interference along the course of their normal reproduction. It is not surprising then that the two earliest theoretical accounts of the von Magnus phenomenon postulate a mechanism resembling that of heterologous interference. The simultaneous infection of a cell by two or more particles, as a prerequisite of incomplete virus production, is the common feature of the hypotheses of von Magnus (1947 ; 1951*a* ; 1951*b* ; 1952) and of Gard (1952). In the extensive studies of von Magnus and his associates this requirement of multiple infection was satisfied, and for this reason his hypothesis appears adequate. Yet, one must not lose sight of some technical peculiarities of the experiments on which this explanation was based.

If a great majority of available cells is infected by the original inoculum, it is immaterial at what time the fluids are harvested as one will always deal with the yield of the first cycle, slightly contaminated perhaps by whatever has been produced in the minority of cells, *viz.*, those that have escaped infection at first. If incomplete particles are found in such experiments, it is safe to infer that they descended directly from the initial seed. The situation is quite different if the number of particles is only equal to or less than the number of infectible cells. Then a fraction—perhaps a small minority only—of cells will be infected at first. Their output will come into contact with large numbers of non-infected cells, and will have an opportunity to go through a second, perhaps a third cycle of multiplication. Obviously, the *final yield* will say very little about the behaviour of the original inoculum, and no valid inferences can be drawn. The method to follow at low multiplicities is to examine the *first-cycle yield* which, by definition, shows the response to the initial inoculum. This technique has such advantages, even when the host cells are outnumbered by infective particles, that no other should be considered when setting up crucial tests.

It is on this ground that von Magnus' demonstration of the necessity of multiple infection is unacceptable, his technique being unwittingly biased in favour of his own hypothesis. In the experiment designed for the assessment of the smallest inoculum after which the phenomenon still occurred (Experiment 3, von Magnus, 1952), incomplete virus simply *could not* be detected at multiplicities of less than 1, where over 30 per cent of the cells escaped immediate infection. He found that " mainly incomplete virus was produced in embryos following inoculation with undiluted or tenfold diluted seed, while the 10^{-2} and 10^{-3} diluted inocula resulted in the formation of fully active virus ". This is a perfectly correct statement if the yields at 24 hours, at the end of the third cycle, are considered. It is significant, however, that at 10 hours the eggs contained $10^{8.6}$, $10^{8.0}$, $10^{6.9}$ and $10^{5.7}$ ID_{50} after inocula of $10^{8.9}$, $10^{7.9}$, $10^{6.9}$ and $10^{5.9}$ infective doses respectively. This lack of change is in contrast with the behaviour of haemagglutinin titres : they rise conspicuously from the 4th hour onwards, as seen with the two larger inocula after which performance of haemagglutinin tests was feasible. This means, obviously, that only minute fractions of the first-cycle yields were infective, the bulk consisting of incomplete particles. From the growth curves it is also clear that by the end of the second cycle (at 15 hours) the eggs contained $10^{8.1}$, $10^{8.9}$, $10^{9.7}$ and $10^{9.5}$ infective doses respectively. Thus after smaller infective inocula a distinct second cycle has occurred,

and there can be no doubt that the virus produced in this cycle was fully infective. The quantitative data also show that the first-cycle yields of the two smaller inocula were completely masked by the produce of later cycles, the infectivity titres rising by a factor of 100 and 7000 between the 10th and 24th hour for the 10^{-2} and 10^{-3} inocula. For the undiluted and 10^{-1} seeds the titres show a 2-fold drop and a 3-fold rise respectively, neither of which can be regarded as significant change.

Recognition of such a technical flaw does not, of course, invalidate the hypothesis ; it merely deprives it of what was regarded as its crucial proof. The demonstration by Cairns and Edney (1952), on the other hand, is in itself enough to discount any theory based on interference. In an elegant experiment, exemplary both in design and accuracy, they were able to show that incomplete virus was being produced at multiplicities as low as 0·02, *i.e.*, where only 2 per cent of the cells had been infected. It adds to the value of these tests that they were done with the PR8 strain, that used by von Magnus.

That multiplicity of infection is not a sufficient cause for the production of incomplete virus should be evident also from the fact that no non-infective particles are formed after infections with dilute seed. Yet, however small the initial inoculum, from the time when more than one-fiftieth of the cells have already yielded, the conditions are those of multiple infection. As each cell produces roughly one hundred new particles, at least half of the cells will be infected by two or more particles in the last cycle. This point is emphasized by our finding of strains where multiple infection even by the initial inoculum does not give rise to non-infective particles.

It is implicit in the auto-interference theory of von Magnus and explicitly stated in the theoretical treatment of Gard that more concentrated inocula, by virtue of giving higher multiplicities of infection, should give issue to a smaller number of complete virus particles than more dilute inocula. The observations of von Magnus, if examined from this point of view, do not support his own hypothesis but appear to be identical with those of Cairns and Edney, who showed that a thousandfold change in the initial inoculum of PR8 virus was not followed by *any* significant variation in the amount of infective particles produced. Our own experiments show that this behaviour applies to influenza strains generally.

The main finding of the present study, namely the gradation in incomplete virus production among influenza strains could be accounted for by the auto-interference theory only with the greatest difficulty. At least two subsidiary assumptions would have to be made : first, that the mechanism was based not on simultaneous infection by two active particles, but rather by an active and an inactive one ; and second, that the various strains contained different amounts of inactive particles, even when grown from dilute seed. However, both of these assumptions would be at variance with observed fact. The first is untenable on quantitative grounds, and would imply increasing effect on increasing the initial inoculum, which is not the case. Or it could be upheld by the further postulate of such a close and regular association between active and inactive particles that defied randomness after inoculation. There is no evidence that anything like this happened ; indeed, the fact that dilute inocula do not produce incomplete particles all but rules it out of consideration. The second assumption appears unjustifiable in the light of the finding that the initial ID/HA ratio of all strains is, for practical purposes, identical while there is more than a hundredfold difference in the degree of incomplete virus production. Also, there is no evidence that

fresh virus preparations grown from dilute seed would contain non-infective particles. Up till recently this has been the expressed opinion of von Magnus (1951*a*), and we have published several points of independent evidence to support this view (Fazekas de St.Groth and Cairns, 1952).

The third hypothesis (Cairns and Edney, 1952) came from an entirely different approach. Indeed, it cannot be called merely a hypothesis but rather an interpretation of experimental facts which rendered earlier hypotheses untenable and pointed in a direction along which a valid theory might be built from material provided by future research. In brief, Cairns and Edney observed that incomplete virus was being produced by inocula which caused negligible (less than 1 in 10,000) multiple infection. Realizing that this happened *only* in the first cycle of multiplication, they postulated that an uninfected egg contained a limited amount of some unknown substance and this set an upper limit to the number of infective virus particles that could be produced immediately. The process of viral multiplication was supposed to stimulate the infected tissue to provide more of this essential substance, and thus in the second and later cycles all of the virus produced might be infective. No attempt was made to define either this substance or the process by which it became available in later cycles. This explanation can account for all available data on incomplete virus production, such as its occurrence without multiple infection of cells, its quantitative independence of the primary inoculum at increasing multiplicities, and also the fact that the von Magnus phenomenon occurs only in the first cycle of multiplication. It should be added that it is consistent also with our findings, and should serve therefore as the basis of their interpretation.

The factual results of our study may be summed up in two points :

1. All influenza strains that produce non-infective particles in the allantois do so above a characteristic level of first-cycle infection. This level is independent of the multiplicity of infection, and is an inherent property of the virus, as are its antigenic make-up, enzyme activity, etc. The proportion of infective particles in the yield cannot be reduced by increasing the initial inoculum. Strains which do not produce incomplete virus fail to do so even at average multiplicities of infection of 10 or higher.

2. The different influenza virus strains form a gradient according to their capacity to produce non-infective particles. This gradient cuts across the line dividing the types, and is independent of the history of the strains as well as of the extent of their adaptation to the allantois.

The first of these points is the confirmation and extension of Cairns and Edney's thesis, which can now be regarded as a general statement holding for all influenza virus strains. The second point, on the other hand, requires the amplification of their hypothesis and its statement in more specific terms.

The variation in the extent of incomplete virus production among the strains could be satisfied by the assumption that their requirements for the " missing factor " are unequal. Yet, operationally this further assumption would be of little use as it gives no lead whereby the mechanism underlying these differences could be better understood ; neither does it suggest an experimental approach simpler than the random testing of all conceivable substances for their effect on incomplete virus production. It might be more profitable then to think in terms of a " missing process " rather than a " missing substance ". In this respect it is noteworthy that the gradient of incomplete virus production corresponds

closely to the gradient of enzyme action of these viruses on the allantoic membrane or, which is saying the same, the order in which infection can be prevented by the use of the " receptor destroying enzyme " of *V. cholerae* (Stone, 1947, 1948).

Whether this view is correct or not, it has the merits of a working hypothesis inasmuch as it immediately prompts several types of experiment by which its validity can be tested. It implies both that the " incomplete virus gradient " should be different in tissues which have different receptor gradients (*e.g.*, allantoic membrane and mouse lung), and that agents acting on receptors might alter the behaviour of influenza strains even in the allantois. An investigation on these lines will be reported later.

One more point calls for some discussion since our observations have a close bearing on it : it is the prolonged simultaneous passage of two influenza strains in the allantois and in mouse lungs. First described by Sugg and Magill (1948) and further investigated by Sugg (1951) and Liu and Henle (1951), the phenomenon appeared highly paradoxical, as from consideration of both cycle times and cyclic increments of influenza A and B strains, one would have postulated a rapid suppression of the B virus by the more quickly growing A strains. This indeed happens when dilute inocula of the two viruses (LEE and PR8) are used, even if the B started at a majority of 5 to 1 (Liu and Henle, 1951) ; yet by keeping the inoculum at the level of 10^7–10^9 particles the mixture could be carried through 52 consecutive passages (Sugg, 1951). The interpretation offered, namely that the cycle time of the LEE(B) strain was considerably shortened after large inocula (Liu and Henle, 1951), is not entirely satisfactory as another A strain " CC " is capable of suppressing LEE even when large initial doses are applied (Sugg and Magill, 1948). Moreover, although the apparent cycle time of LEE is shortened, its cyclic increment is not increased under the same conditions, as evident from Liu and Henle's published data (Fig. 4, 5, 6),

A simpler explanation can be offered now on the grounds of our findings. The slower growth rate of the LEE strain is compensated for by the large fraction of non-viable offspring the PR8 strain produces after concentrated inocula. The CC strain, producing a smaller amount of incomplete virus, will readily outgrow the LEE strain whether starting at high or low dilutions. It is significant that this CC versus LEE behaviour applies *only* to the allantois and not to mouse lungs, where the two viruses could be carried through five passages without any diminution of their initial ratio, and through further passages with only slight drop of the LEE component (Sugg and Magill, 1948). This latter difference is in agreement with the postulate of our working hypothesis that the amount of incomplete virus produced should vary from tissue to tissue.

SUMMARY.

Ten representative influenza virus strains have been tested for production of non-infective elementary bodies after allantoic inoculation of concentrated seed. The strains were found to differ widely in their capacity to yield incomplete offspring, and to fall into the gradient PR8(A) > WSE(A), MEL(A) > SW > BON(B), BEL(A) > HUT(B), CAM(A′) > FM1(A′) > LEE(B), in the order of decreasing incomplete virus production.

By serial passaging of undiluted inocula it could be shown that even the strains at the lower end of the gradient produced some non-infective particles under special conditions.

The hypotheses covering incomplete virus production are discussed in detail, and a new interpretation is offered to account for the serial simultaneous passage of two influenza strains in various hosts.

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THE INFLUENCE OF CHEMICAL CONSTITUTION ON ANTI-BACTERIAL ACTIVITY. PART VII: THE SITE OF ACTION OF 8-HYDROXY-QUINOLINE (OXINE).

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It has been shown that 8-hydroxyquinoline is not toxic for *Staphylococcus aureus* unless traces of iron (or copper) are present in the medium (Rubbo, Albert and Gibson, 1950). It was next shown that a large excess of either iron or oxine prevented a rapid bactericidal action. This led to the hypothesis that oxine gained access to the cells as a complex having one atom of the metal bound between two molecules of oxine (to be referred to briefly as the 1 : 2 complex (XIII)); and that it exerted its toxic effect inside the cells in the form of the unsaturated complex which consists of one molecule of oxine bound to one atom of the metal (*i.e.*, the 1 : 1 complex (XII)) (Albert, Gibson and Rubbo, 1953).

It will be noted that this hypothesis postulates that oxine acts within the cell, and not on the outside as the aminoacridines do (Albert, 1951). The present work was carried out to test that assumption by varying the lipophilic nature of oxine by means of suitable substituents in the molecule. It is generally agreed that in a series of related substances the ease of penetration through a cell membrane is proportional to the lipophilic nature of the substance (Davson and Danielli, 1943; Höber, 1945). This lipophilic nature can be conveniently measured in terms of oil/water partition coefficients.

In the present work the coefficient of oxine has been lowered by the insertion of one or more ring-nitrogen atoms, and the coefficients of some of the resulting substances have been raised again by the insertion of alkyl groups. It will be shown that the antibacterial properties disappear as the coefficient is lowered, and re-appear when it is raised again. These facts provide support for the hypothesis that oxine acts inside the cell.

EXPERIMENTAL.

Bacteriology.

The antibacterial activities of the substances described below were assessed by finding the greatest dilution that would prevent visible growth. In order to preserve continuity with previous studies in this series, these tests were performed with the six species of bacteria used previously (Albert, Rubbo, Goldacre and Balfour, 1947). The strains of Gram-positive bacteria used (*Clostridium welchii*, *Streptococcus pyogenes* and the Oxford strain of *Staphylococcus aureus*) were not the same as in the earlier series and under the conditions of the tests were about 4 times less sensitive to oxine. Hence oxine was included as a control substance in all experiments so that a relative assessment of the antibacterial action of the new substances could be made in spite of differences in the strains used and in the conditions of the experiments. In the final experiment (Table II), another strain of *Str. pyogenes*, equal in sensitivity to the original strain, was introduced. The Gram-negative strains used

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(*Bacterium coli, Proteus, Pseudomonas pyocyanea*) were similar in sensitivity to the original strains.

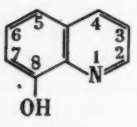
The bacteriostatic tests consisted of inoculating a series of twofold dilutions of the substance in broth (pH 7·3) and inspecting the solutions for evidence of growth after 48 hr. incubation at 37°. Each substance was tested in duplicate. The solutions of the test substances (M/800 in broth) were autoclaved at 15 lb. for 15 min. (120°) in an air-tight screw-capped container. From this stock solution, serial dilutions were prepared in autoclaved broth.

The metal-depleted broth used for the experiment shown in Table III was prepared by repeated extraction of broth with oxine and chloroform as described by Rubbo *et al.* (1950). In this experiment fourfold serial dilutions of oxine (or its analogue) were made in oxine-treated broth and in untreated broth, and then distributed in 2 ml. amounts. Solutions of $FeSO_4 7H_2O$ (Analar) and $CoSO_4 7H_2O$ (Analar) were made in treated and untreated broth at a concentration of M/12,500. These metal solutions were added in 1 ml. amounts to sets of serial dilutions of oxine as required, and the volume of each tube was made up to 4 ml. with the appropriate broth where necessary. Control tubes containing the metals without oxine were also set up. The tubes were then inoculated with 3 drops of a 24-hr. culture of the Oxford staphylococcus in oxine-treated broth and the results were read after incubation for 18 hr.

Chemistry.

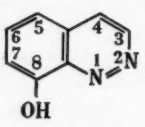
The ionization constants, stability constants and partition coefficients are taken from Albert and Hampton (1954). The substances were prepared as described by Albert and Hampton (1952, 1954). These substances are described uniformly as aza-oxines, using the same numbering as for oxine (I). Alternative names also exist for them and have been placed under each formula to assist indexers.

FORMULAE.



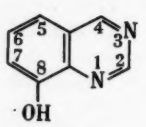
Oxine

(I)



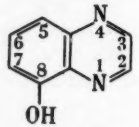
2-Aza-oxine
(8-Hydroxycinnoline)

(II)



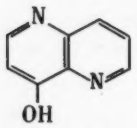
3-Aza-oxine
(8-Hydroxyquinazoline)

(III)



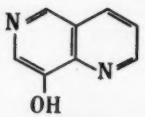
4-Aza-oxine
(8-Hydroxyquinoxaline;
5-Hydroxyquinoxaline)

(IV)



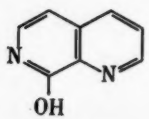
5-Aza-oxine
(4-Hydroxy-1 : 5-naphthyridine)

(V)



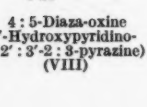
6-Aza-oxine
(8-Hydroxy-1 : 6-naphthyridine)

(VI)



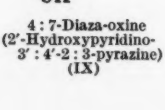
7-Aza-oxine
(8-Hydroxy-1 : 7-naphthyridine)

(VII)



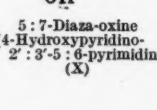
4 : 5-Diaza-oxine
(4'-Hydroxypyridino-2' : 3'-2 : 3-pyrazine)

(VIII)



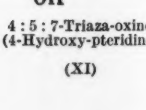
4 : 7-Diaza-oxine
(2'-Hydroxypyridino-3' : 4'-2 : 3-pyrazine)

(IX)



5 : 7-Diaza-oxine
(4-Hydroxypyridino-2' : 3'-5 : 6-pyrimidine)

(X)



4 : 5 : 7-Triaza-oxine
(4-Hydroxy-pteridine)

(XI)

It will be seen that the substances first investigated are analogues of oxine carrying one extra ring-nitrogen in every possible position (II to VII). Others carry two or three extra ring-nitrogens (VIII to XI). These form No. 2–11 of Table I. The insertion of even one ring-nitrogen greatly decreases the lipophilic properties of (I), as Table I shows.

Later, homologues of (II) and (III) were prepared in which a methyl ($-CH_3$), propyl ($-CH_2CH_2CH_3$) or allyl ($-CH_2CH:CH_2$) side-chain was included for the purpose of restoring lipophilic properties. These syntheses produced 4-methyl-2-aza-oxine (4-methyl-8-hydroxycinnoline), 4-methyl-3-aza-oxine (4-methyl-8-hydroxyquinazoline), 4-propyl-3-aza-oxine and 7-allyl-3-aza-oxine (No. 17–20). Their formulae can be easily visualized with the help of numbering included in formulae (II) and (III).

All of the above substances form tightly bound complexes with the ions of metals by the process of chelation, *i.e.*, by building the metal ions into a new heterocyclic ring, *cf.* (XII). Some non-chelating isomerides of the above substances were prepared as controls. These (No. 12–16) are not capable of chelation because their hydroxyl-group is not *peri* to a ring-nitrogen.

RESULTS.

Table I gives the results of the bacteriostatic testing of oxine, its aza-derivatives and some non-chelating isomerides of the latter. It is evident that the mono-aza-oxines fall into two sub-classes, (*a*) those having a partition-coefficient about one-tenth of that of oxine (No. 2, 3, 4); and (*b*), those having a still lower coefficient (No. 5, 6, 7). The antibacterial action of (*a*) is less than that of oxine, but quite appreciable, the antibacterial action of (*b*) is much less. The poly-aza-oxines (No. 8–11) resemble group (*b*), having low partition coefficients and lacking antibacterial activity.

The substances No. 12–16 were synthesized to provide a series of isomerides of No. 2–4. These differ only in that they do not chelate with metallic ions. Thus No. 12 is isomeric with No. 2, No. 13–14 with No. 3, and No. 15–16 with No. 4. It is evident that none of these non-chelating isomerides have the antibacterial powers of their chelating prototypes.

TABLE I.—*Bacteriostatic Action of Oxine and Related Substances.*

Highest dilutions (expressed as 1/M) completely preventing visible growth in 48 hr. at 37° (medium: broth, pH 7·3).

No.	Substance.	Gram-positive organisms.			Gram-negative organisms.			Partition coefficient (oleyl alcohol/water).
		Cl. welchii.	*Str. pyogenes.*	*Staph. aureus.*	*Bact. coli.*	*Proteus.*	*Ps. pyocyanea.*	
1	Oxine	25,600	12,800	25,600	1,600	1,600	—	67
	Mono-aza-oxines:							
2	2-Aza-oxine	1,600	800	800	—	—	—	6
3	3-Aza-oxine	3,200	1,600	1,600	—	—	—	5
4	4-Aza-oxine	3,200	800	800	—	—	—	8
5	6-Aza-oxine	1,600	—	—	—	800	—	1
6	7-Aza-oxine	—	—	—	—	—	—	0·14
7	5-Aza-oxine	800	—	—	—	—	—	< 0·02
	Poly-aza-oxines:							
8	4 : 5-Diaza-oxine	—	—	—	—	—	—	< 0·02
9	4 : 7-Diaza-oxine	—	—	—	—	—	—	
10	5 : 7-Diaza-oxine	—	—	—	—	—	—	
11	4 : 5 : 7-Triaza-oxine	—	—	—	—	—	—	
	Non-chelating isomerides of No. 2–4:							
12	4-Hydroxycinnoline	—	—	—	—	—	—	
13	4-Hydroxyquinazoline	—	—	—	—	—	—	
14	6-Hydroxyquinazoline	—	—	—	—	—	—	
15	2-Hydroxyquinoxaline	—	—	—	—	—	—	
16	6-Hydroxyquinoxaline	—	—	—	—	—	—	

— Signifies growth at M/800. It is not convenient to use solutions stronger than M/800 because of poor solubility.

In order to provide a series of mono-aza-oxines which would be more lipophilic than No. 2–4, substances were synthesized similar to these but having, in addition, a short aliphatic side-chain in order to increase the partition coefficients. As it was evident from preliminary tests that these new substances (No. 17–20) were giving results of considerable theoretical interest, special care was used in designing the biological test, the results of which are reported in Table II. All the substances mentioned in this table were tested at the same time, whereas those in Table I were done in batches of five or six (always including oxine as a control). As has been mentioned, the strain of *Str. pyogenes* used in this experiment was more sensitive than that used in the earlier tests; in the meantime, the Oxford staphylococcus had apparently become more resistant in that there was a greater tendency for subsequent growth to appear in tubes showing bacteriostasis at 24 hr.

Besides the new alkyl-substituted aza-oxines (No. 17–20), it was decided to include in this test not only oxine, but all the aza-oxines which had shown any antibacterial activity in Table I. It will be seen that for substances common to Tables I and II, the results for *Cl. welchii* agree to within one tube, which is what is to be expected for this type of test. The results with *Str. pyogenes* are naturally higher in Table II, but even the use of the more sensitive strain has not elicited any activity from No. 7. The results obtained with *Staph. aureus* lie so near the bottom of the scale, that comparison with Table I is difficult.

It is evident from Table II that the alkylated aza-oxines (No. 17–20) have higher partition coefficients than their lower homologues, No. 2 and 3. In fact in two cases the coefficients surpass that of oxine. It is also evident that the antibacterial activity of these substances surpasses that of the unsubstituted aza-oxines (No. 2–7), but even in No. 20 it remains a little short of that of oxine (*i.e.*, by one tube for each organism).

Apart from these three Gram-positive organisms, all the substances in Table II were tested simultaneously against the three Gram-negative organisms listed in Table I. However, little or no activity was found, a feature typical of the 8-hydroxyquinolines as a class (Albert *et al.*, 1947). It was thought, however, that more specific tests were desirable to show that the mode of action of the alkylated compounds was of the same nature as that of oxine. The results of such tests (Table III) show that the action of representative alkylated aza-oxines is biologically similar to that of oxine.

In order to discover the relative metal-binding powers of the various chelating substances in Tables I and II, potentiometric titrations were carried out in the

TABLE III.—*Comparison of the Bacteriostatic Properties of Oxine and Representative Alkylated Aza-oxines.*

Highest dilutions (as 1/M) completely preventing visible growth of *Staph. aureus* at 37° (pH 7·3) after 18 hr.

No.	Substance.	Nutrient broth (as used in Table II).	Metal-depleted broth. Without additions.	Plus— M/50,000 $FeSO_4$.	Plus— M/50,000 $CoSO_4$ M/50,000 $FeSO_4$.
1	Oxine	>100,000	1,600	>100,000	25,000
18	4-Methyl-3-aza-oxine	6,400	<1,600	>100,000	25,000
20	4-Allyl-3-aza-oxine	6,400	1,600	>100,000	25,000

TABLE II.—*Bacteriostatic Action of Oxine and Related Substances* (continued).

Highest dilutions (expressed as 1/M) completely preventing visible growth in 48 hr. at 37° (medium : broth, pH 7·3). Partition coefficients, stability constants and $\bar{n}$ values.

No.	Substance.	Organisms. *Cl. welchii.*	Organisms. *Str. pyogenes.*	Organisms. *Staph. aureus.*	Average bacteriostatic titre.	Partition coefficient (oleyl alcohol/water.)	Stability of 1 : 2 complexes (log K_2). Fe+++.	Cu++.	Ni++.	Fe++.	Average no. of mols. bound by one metallic cation (pH 7·3 and M/5000)[c]. Cu++.	Ni++.	Fe++.
1	Oxine	25,600	200,000	6,400	77,000	67	33	23	18	15	2·0	1·85	1·4
	Mono-aza-oxines :												
2	2-Aza-oxine	3,200	12,800	800	5,600	6	26	17	15	12·5	2·0	1·9	1·7
3	3-Aza-oxine	1,600	12,800	—	4,800	5	28	17·5	14·5	12	2·0	1·8	1·5
4	4-Aza-oxine	3,200	6,400	—	3,200	8	26	*b*	14·5	12·5	*b*	1·8	1·6
5	6-Aza-oxine	3,200	1,600	800	1,900	1	26	14	11	10·5	1·0	0·9	0·9
7	5-Aza-oxine	800	—	—	270	<0·02	28	13·5	10·5	10·5	0·8	0·2	0·2
	Alkylated Mono-aza-oxines :												
17	4-Methyl-2-aza-oxine	6,500	25,600	6,500	13,000	16	*b*	*b*	15	*b*	*b*	1·8	*b*
18	4-Methyl-3-aza-oxine	6,500	51,200	3,200	20,000	17	*b*	*b*	14·5	*b*	*b*	1·9	*b*
19	4-Propyl-3-aza-oxine	12,800	100,000	1,600	38,000	135	*b*	*b*	14·5	*b*	*b*	1·8	*b*
20	7-Allyl-3-aza-oxine	12,800	100,000	3,200	39,000	310	*b*	*b*	14·5 *a*, *b*	*b*	*b*	1·8	*b*

— Signifies growth at M/800. *a*. From analogy with No. 3, 18 and 19. *b*. Complex is too insoluble to measure.

c. Referred to in text as $\bar{n}$ (the maximum possible value of $\bar{n}$ is 2·0).

presence of the following cations: Fe^{+++}, Cu^{++}, Ni^{++} and Fe^{++} (Albert and Hampton, 1953). From these titrations, the overall stability constant (K_s) was calculated for each complex. This constant (K_s) is a measure of the mass action equilibrium between the metallic cations and the anions of the chelating substances (on the one hand) and the 1 : 2 complexes (on the other). The greater the affinity between the ions, the more stable is the complex, and the higher the K_s. This overall stability constant is the product of two partial stability constants, of which K' governs the formation of the 1 : 1 complex, whereas K'' governs the formation of the 1 : 2 complex from the 1 : 1 complex (in the presence of a further supply of chelating substance). Thus $K_s = K' \,.\, K''$; hence $\log K_s = \log K' + \log K''$. (In the case of trivalent iron, $K_s = K' \,.\, K'' \,.\, K'''$ because a 1 : 3 complex is formed).

The reason for including nickel in these studies is as follows: In carrying out this work, precipitates were often encountered when titrating the iron and copper complexes, and this often prevented values being obtained directly for K_s (when K' is known, the overall stability constant can be derived indirectly, from the approximation: $\log K_s = 2 \log K' - 1$). The generally greater solubility of the nickel complexes almost always allowed complete titration curves to be obtained, so that the oxine-like character of the chelation process could be inspected. As the figures in Table II show, the constants for nickel tend to lie between those for Fe^{++} and Cu^{++}. In some cases the insolubility of the iron and copper complexes was so great that no figures for K' could be obtained: in such cases, the availability of constants for nickel provided useful information on the relative metal-binding powers of the various substances.

It was thought desirable to know not only the stability constants, but also the $\bar{n}$ values, *i.e.*, the average number of molecules of substance bound by one atom of metal under the experimental conditions ($\bar{n}$ varies with K_s, dilution and pH, whereas K_s is independent of $\bar{n}$, dilution and pH). To calculate $\bar{n}$ from K_s, two new equations have now been evolved by reversing the usual derivation of K_s from experimentally determined values of $\bar{n}$ (Albert, 1950, 1952). These equations are simplified to suit the case where, as here, a 1 : 2 ratio exists between the concentration of the metallic salt and that of the metal-binding substance. Equation (i) is for values of $\bar{n}$ between 0 and 1; equation (ii) gives values between 1 and 2. As these are quadratic equations, two answers are obtained, one rational (lying between the above limits) and one absurd (*e.g.*, 297). Unless (i) gives a value that is approx. 1, there is no need to solve equation (ii).

$$n = \frac{3[M^\circ]K' + \alpha \pm \sqrt{[M^\circ]^2(K')^2 + 6\alpha[M^\circ]K' + \alpha^2}}{2[M^\circ]K'} \quad . \quad . \quad \text{(i)}$$

$$\bar{n} = \frac{4[M^\circ]K'' + \alpha \pm \sqrt{\alpha(4[M^\circ]K'' + \alpha)}}{2[M^\circ]K''} \quad . \quad . \quad . \quad . \quad \text{(ii)}$$

where

$[M^\circ]$ is the concentration of metal, free and combined,
K', K'' and $\bar{n}$ have the meanings given above,

$$\alpha = \frac{[H^+]^2}{K_a K_a'} + \frac{[H^+]}{K_a} + 1$$

(where K_a is the higher (and K_a' the lower) acidic ionization constant).

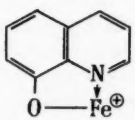
Several $\bar{n}$ values are included in Table II. The dilution of M/5000 has been chosen as an intermediate value within the range of limiting bacteriostatic dilutions ($\bar{n}$ tends to decrease on dilution). It can be found, from equations (i) and (ii), that two substances may have different stability constants and still give the same value of $\bar{n}$ under experimental conditions, provided that the substance with the smaller K_s is compensated by having a larger K_a (a larger K_a implies a smaller pK_a). Because ring-nitrogens promote the ionization of acids, it is not surprising that several of the aza-oxines are compensated in this way (Albert and Hampton, 1953).

It is evident that Table II contains seven substances which combine with metals as effectively as oxine but which have partition coefficients covering a wide range (from 5 to 310, oxine being 67). Thus it is clear that a series of substances has been synthesized having the physical properties requisite to test the hypothesis.

DISCUSSION.

The present work was undertaken to obtain evidence as to whether the action of oxine could take place inside the bacterial cell. Oxine itself is not toxic to staphylococci unless traces of iron (or copper) ions are present. The fact that the concentration of these ions relative to that of the oxine is critical, led to a postulate that the mode of action was inside the cell (Albert *et al.*, 1953). It is desirable now to review the evidence for this postulate. The hypothesis, of which it forms an essential part, is that the 1 : 1 complex (XII) is the actual toxic substance (likely, because it is unsaturated), but that it cannot penetrate the cell (likely, because (XII) is ionized and hence not lipophilic); oxine enters the cell as such and as the 1 : 2 complex (XIII) (likely, because they are highly lipophilic); this 1 : 2 complex is non-toxic (likely, because it is saturated); in the absence of excess oxine inside the cell, the 1 : 2 complex is broken down to the toxic 1 : 1 complex and death results; in the presence of excess oxine, rapid death does not occur because the 1 : 2 complex persists in the cell and is harmless; in the presence of excess iron, rapid death does not occur because no 1 : 2 complex can be formed, according to the law of mass action.

FORMULAE.



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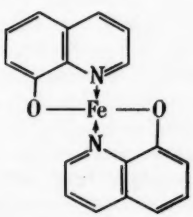

(XIII)

One piece of evidence was offered (by Albert *et al.*, 1953) in support of action being confined to the inside of the cell, namely that 8-hydroxyquinoline-5-sulphonic acid, although its complexes have the same stability constants as oxine, is not antibacterial (neither this acid nor any of its complexes is liposoluble). A possible objection to this evidence is that this acid and all of its complexes

must carry a strong negative charge (on the sulphonic acid group), and hence they do not belong to the same charge-type as oxine and its complexes. Thus they may conceivably be repelled from the surface of bacteria by a coulombic effect.

To meet this possible objection, a series of analogues of oxine has been synthesized in which lipophilic properties are progressively lowered by the insertion of extra heterocyclic nitrogen atoms; later, other substances with increased lipophilic properties have been synthesized by the insertion of small alkyl groups into these aza-oxines. Some of these new substances are even more lipophilic than oxine. These operations do not change the charge-type from that of oxine. If action takes place inside the cells, it would be expected to fall off with decreasing oil/water partition coefficients, which are believed to be a measure of the ease with which a substance penetrates the cell membrane (Davson and Danielli, 1943; Höber, 1945). Conversely, antibacterial action should be restored when the substances with low coefficients are chemically modified so that their coefficients approach that of oxine. To what extent these expectations have been realized will now be discussed.

As can be seen from Table I, the mono-aza-oxines (*i.e.*, oxines with one extra ring-nitrogen) have various partition coefficients, all much lower than that of oxine. These substances are all much less antibacterial than oxine, and the loss of antibacterial properties is, on the whole, greatest in those with the lowest coefficients. Further, the di- and tri-aza-oxines (No. 8–11) have partition coefficients so low that they could not be measured, and they all lack antibacterial activity.

An important step in the discovery that oxine owes its antibacterial activity to chelation was the examination of the six hydroxyquinolines isomeric with oxine (Albert *et al.*, 1947). None of these isomers has a ring-nitrogen atom in the *peri* position relative to the hydroxyl group: hence it cannot bind metals by chelation. It was recognized as significant that none of these isomers was antibacterial. Hence it was thought important in the present studies to examine isomers of the antibacterial mono-aza-oxines. Hence isomers (No. 12–16) of 2-, 3- and 4-aza-oxine were prepared. For the structural reasons just discussed none of these substances can chelate with metallic ions (this lack of affinity for metallic ions was verified experimentally). It is evident from Table I that none of these substances is antibacterial. Hence it seems certain that what activity the mono-aza-oxines possess is due to their chelating ability, just as with oxine.

At this stage it should be explained why oleyl alcohol was used in determining the oil/water partition coefficients. It has been shown that the distribution of a series of substances between water and various poorly miscible liquids always follows the same order, although the less water-soluble liquids give the greater spread of values (Collander, 1947). Oleyl alcohol was used in preference to vegetable oils in the present studies because it is less viscous and more readily purified. It is a representative of the fatty alcohols found in cytoplasmic membranes.

To see if raising the partition coefficient would restore antibacterial activity to the aza-oxines, some homologues were synthesized of two aza-oxines (No. 2 and 3) which had shown moderate antibacterial activity. These homologues (No. 17–20) differed only in the possession of an alkyl group containing from one to three carbon atoms. The results of this investigation are given in Table II, and the opportunity was taken to test, *at the same time*, all substances from Table I which had shown activity (see under "Results" for comments on the strains of organisms used).

It is evident from Table II that the device of inserting small alkyl groups has been successful in creating a range of mono-aza-oxines having increased partition coefficients (the two highest exceed that of oxine). Simultaneously, the antibacterial action has increased until it is almost as great as that of oxine (these tests are conducted with two-fold dilutions ; thus No. 20 is consistently one tube less active than oxine). It is evident that the antibacterial action of oxine has been decreased, and even extinguished, by altering the molecule so as to lower the oil/water partition coefficient, and then almost completely restored by further alterations which increase the coefficient. In this connection it is interesting to recall that the antibacterial activity of oxine itself is not improved by increasing the partition coefficient which seems to be optimal (5-methyl- and 5-propyl-oxine have about the same antibacterial activity as oxine (Albert *et al.*, 1947)).

The question now arises whether in altering the chemical structure of oxine so as to vary the partition coefficients, the metal-binding powers of oxine were also being decreased, thus introducing a second variable. Investigation of the data provided in Table II shows that this has indeed taken place in the case of No. 5 and 7, but that there are seven other substances which have metal-binding powers of the same order as those of oxine, and yet vary widely in partition coefficient and antibacterial action. These substances are No. 2, 3, 4, 17, 18, 19 and 20. The basis of comparison is the $\bar{n}$ value (given in the last columns of Table II), *i.e.*, the average number of molecules of the substance bound by one atom of metal. (The special suitability of nickel as a reference metal has been discussed under "Results," where it is also shown how two substances having different values of K_s can have the same value of $\bar{n}$).

It was important to discover whether the mode of action of the highly effective alkylated aza-oxines was of the same nature as that of oxine. Oxine has the following characteristic biological properties : it shows (i) reduced antibacterial activity in metal-depleted broth, (ii) full antibacterial activity in metal-depleted broth to which a trace of a ferrous salt has been added, and (iii) substantial antagonism of the increase described under (ii) when a cobalt salt is added at the same concentration as the iron (Albert *et al.*, 1953). It is evident from Table III that the alkylated aza-oxines show all these effects and hence are exerting their toxic action through a mechanism similar to that of oxine.

The hypothesis outlined at the beginning of this discussion demands free access to the cell of both oxine and its 1 : 2 complexes. Hence not only should oxine and its analogues be lipophilic to be effective (as has been demonstrated above), but the 1 : 2 complexes should be lipophilic also. That they are even more lipophilic than the metal-free substances follows from theoretical grounds because the 1 : 2 complexes have fewer centres of hydration (the 1 : 1 complexes, being ions, are highly hydrated). It would be most satisfactory if partition coefficients for the 1 : 2 complexes could be determined experimentally, but this is difficult because the metals can only be held entirely as 1 : 2 complexes when an excess of the metal-free substance is present (law of mass action), and its presence would vitiate the calculations. A satisfactory compromise is to find whether the aza-oxines are more completely extracted by oil from water when metallic ions are present and if the most lipophilic aza-oxines are those most completely removed from water in this way : both of these expectations have been realized experimentally (Albert and Hampton, 1954).

The most biologically active substance to emerge from this work is 7-allyl-

3-aza-oxine (No. 20), and it was thought of interest to see whether it had chemotherapeutic powers in the mouse. The $L.D._{50}$ (subcutaneous) was found to be $> 0{\cdot}2$ g./kg., and hence it is considerably less toxic than oxine (0·03 g./kg., on the same strain of mice). Hence it has a higher *in vitro* chemotherapeutic index than oxine. However, as with oxine, no chemotherapeutic effect was demonstrable in mice (infected with *Str. pyogenes*, Richards strain) when 50 mg./kg. was injected with or without 5 mg./kg. of copper sulphate. Moreover its *in vitro* bacteriostatic effect was strongly lowered in the presence of intact red blood cells. Oxine, also, has little antibacterial action in the presence of red blood cells (Rubbo *et al.*, 1950). It is known that the partition coefficient of oxine between red blood cells and normal saline is 7, which does not seem unduly high (Shaw, 1928). Whether these cells merely withdraw oxine (and its analogues) from circulation, or whether they chemically alter it, *e.g.*, by conversion to glucuronate or sulphate (which would be non-chelating and hence inactive), would be information worth acquiring.

SUMMARY.

To obtain further evidence for the site of action of 8-hydroxyquinoline (oxine), analogues were examined having from one to three extra ring-nitrogen atoms. These substances covered a range of oil/water partition coefficients, all of which were lower than that of oxine. It was found that, as the coefficients fell, the antibacterial activity also fell, and tended to disappear.

Representative examples of these aza-oxines were then structurally modified by inserting small alkyl groups so that the partition-coefficients increased and eventually exceeded that of oxine. It was found that concomitant with this restoration of high coefficients, an antibacterial activity almost equal to that of oxine was realized.

These new substances (of which 7-allyl-3-aza-oxine is the most active) exert their antibacterial activity by the same mechanism as oxine. Like oxine, they are less active in metal-depleted broth, their activity is restored on the addition of iron, and it can then be antagonized by cobalt.

It is concluded that this new evidence provides support for the hypothesis that the site of action of oxine is inside the bacterial cell.

One of us (A. H.) expresses his thanks to the Australian National University for a Scholarship.

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THE INFLUENCE OF STREPTOCOCCAL TOXIN ON THE DEVELOPMENT OF TUBERCULIN SENSITIVITY IN GUINEA-PIGS.

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THE onset of rheumatic fever is commonly, if not invariably, preceded by infection with haemolytic streptococci of Lancefield's Group A (Coburn, 1931), but opinion is divided between those who consider Group A haemolytic streptococci essential to the disease, and others, " the majority " according to Hench and his colleagues (1938) ,who " consider the prodromal respiratory infections to be non-specific provocatives " comparable in action to other stressor agents, such as trauma, cold, fatigue, sunburn or malaria (Waksman, 1949), which it is implied can initiate the disease in the absence of streptococcal infection. However, inaccuracy in bacteriological diagnosis would account for the few claims that rheumatic fever can occur in the absence of streptococcal infection, for even in scarlet fever failure to isolate streptococci occurs in 10 per cent of cases (Wilson and Miles, 1946) ; presumably this figure would be greater 3–4 weeks later at the time when immunity is high, convalescence approaches and the rheumatic syndrome appears.

In spite of the attention devoted to the study of serological responses in patients with rheumatic fever, none are pathognomonic, or indeed distinguishable from those associated with chronic streptococcal infection ; neither has an increased incidence of rheumatic fever been associated with any particular type or strain of Group A streptococcus (Swift, 1949 ; Rantz, Boisvert and Spink, 1945). It is therefore probable that some general property of Group A streptococci initiates the rheumatic syndrome in susceptible persons. But if there is nothing specific in the response to haemolytic streptococci of the rheumatic fever patient, at least certain criteria have to be fulfilled. The infection must persist for approximately 3 weeks ; chemotherapy or antibiotic treatment during this stage prevents rheumatic fever, but later, once the disease is established, such therapy is ineffective (see, *e.g.*, Rantz, Spink, Boisvert and Coggeshall, 1945). Waksman (1949) argues that " the fact that sulphonamide compounds and penicillin are ineffectual when administered after the onset of an attack is additional proof that the initiating infection is not the direct ' cause ' of the disease but acts only by starting in motion some more complex mechanism." But, the ineffectiveness of these drugs in the treatment of the non-pyogenic complications of scarlet fever does not mean that the latter are not induced by streptococci. It might equally well be argued that failure of streptomycin to eliminate tuberculin sensitivity means that such sensitivity is attributable to causes other than infection with the tubercle bacillus. A more probable explana-

tion is that, if a haemolytic streptococcal infection is eliminated in under three weeks, rheumatic fever will not develop, but, if the infection persists after three weeks, non-pyogenic lesions, including rheumatic fever, which do not respond to treatment with antibacterial agents, may result.

The idea that haemolytic streptococci are " stressor agents " or " non-specific provocatives " leaves unexplained the dramatic parallel decrease since 1940 in deaths from haemolytic streptococcal infections and from rheumatic fever (Wheatley, 1949). and implies that either it is unassociated with the advent of antibiotics and chemotherapy or else that penicillin and sulphonamide-sensitive " stressor agents " are of greater significance than insensitive " stressor agents ". But even the idea of " penicillin-sensitive stressor agents " is unacceptable, for if rheumatic fever is due to streptococcal stress, the incidence should be proportional to the severity of the initial attack of streptococcal tonsillitis and presumably occur when stress is maximal rather than 3 weeks later. It does neither of these things ; therefore, an alternative hypothesis must be found.

Bacterial allergy.

Persistent low-grade streptococcal infection in spite of abnormally high antitoxin titres, predisposition of one attack of rheumatic fever to another, a decreasing time interval between infection and recurrence, dermal hypersensitivity to streptococcal allergens (Coburn, 1931 ; Swift, Hitchcock and Derick, 1928), a delayed tuberculin type of reactivity following intravenous injection of streptococcal allergens (Swift *et al.*, 1928), all suggest that rheumatic fever is initiated by hypersensitivity to streptococcal allergens. In keeping with this idea, the histological changes, in contrast to those usually associated with a pyogenic coccus, are characterized by absence of bacteria, extensive oedema, increased capillary permeability, changes in collagen, accumulation of lymphocytes, and absence of polymorphonuclear cells. Relief of symptoms with cortisone therapy, which effectively depresses bacterial hypersensitivity in man but has little if any protective power against non-specific irritation (Lovell, Goodman, Hudson, Armitage and Pickering, 1953), again supports this contention. Less satisfactory evidence is obtained from the ease with which the characteristic histological changes can be mimicked in experimental animals by many different sensitizing processes (see, *e.g.*, Waksman, 1949).

Opinion is divided, among those who consider that rheumatic fever is initiated by hypersensitivity to streptococcal allergens, as to whether such sensitivity is of the tuberculin or of the anaphylactic type. Bacterial allergy of the tuberculin type is a naturally occurring phenomenon typically associated with low-grade persistent foci of comparatively avirulent streptococcal (Angevine, 1939, 1941) or of tuberculous infection ; it takes 3 weeks from the initial infection to develop fully, at which time a delayed inflammatory-necrotizing response occurs on injection of the allergens. Such sensitivity is independent of circulating antibody, but can be passively transferred with cells.

In direct contrast, the anaphylactic type of sensitivity is a laboratory phenomenon, artificially induced by a single injection of a protein ; it takes 10 days to develop, after which time a second injection of protein produces an immediate dramatic response characterized by spasm of smooth muscle. Such sensitivity, associated with circulating antibody, and passively transferable, provides an interesting research tool, although it bears no known relationship to naturally

occurring disease in man. It is probable therefore that the delayed tuberculin type of hypersensitivity to the streptococcal allergens initiates the rheumatic syndrome. But hypersensitivity may develop in the post-streptococcal state without symptoms of rheumatic fever. This is less of a distinction than it at first appears to be, for rheumatic carditis frequently occurs without a history of rheumatic fever, the post-streptococcal state merging imperceptibly into the rheumatic syndrome (Rantz, Spink and Boisvert, 1946); however, though streptococcal allergy may be independent of rheumatic fever, there is no convincing evidence that the reverse is true. It is probable that streptococcal allergy initiates the rheumatic syndrome but that other factors determine the clinical intensity of the disease.

If bacterial allergy of the tuberculin type is essential to the rheumatic syndrome, the tubercle bacillus might be expected to be associated with rheumatic fever almost as frequently as the streptococcus. It is not our purpose to review the evidence that tuberculosis can occasionally initiate both rheumatic fever and the analogous disease, rheumatoid arthritis. We are concerned only with the indisputable fact that the relationship, if it occurs at all, is infrequent. Therefore it seems probable that Group A haemolytic streptococci in general contain not only an allergen but other factors responsible for this difference.

Rheumatic fever is a disease of mesenchymal tissue. The toxins of haemolytic streptococci are mesenchymal toxins, which, compared with bacterial exotoxins in general, are remarkable for their low toxicity. It occurred to us that mesenchymal cells, damaged by streptococcal toxin, might be more sensitive than undamaged cells to a bacterial allergen; that haemolytic streptococci might produce both allergens and toxins which act synergistically upon the same cells. In this paper we provide evidence to support this hypothesis. We show that repeated intramuscular injections of streptococcal toxin increase the speed and intensity of sensitization to tuberculin in guinea-pigs injected with dead tubercle bacilli. The peak of hypersensitivity was reached in the fourth week; animals treated with toxin were more sensitive than the controls. No difference could be detected in the maturation-rate of lesions resulting from injection of tuberculin into toxin- and non-toxin-treated animals. Toxins derived from several different strains of Group A streptococci increased sensitivity; scarlatinal antitoxin administered before the toxin injections prevented this increase, but not if injected later. The toxin was destroyed by heat. Exotoxins derived from bacteria other than haemolytic streptococci did not increase sensitivity. Cortisone proved less effective in depressing hypersensitivity enhanced by streptococcal toxin than hypersensitivity not so enhanced.

METHODS.

Albino guinea-pigs of the Hampstead strain weighing 350–500 g., fed on Bruce and Parkes (1947) pelleted diet supplemented with unlimited cabbage, were sensitized either by intramuscular injection of 1·5 mg. dry weight, heat-killed virulent tubercle bacilli in 0·5 ml. liquid paraffin, or with 2·0 mg. wet weight of B.C.G. suspended in 0·5 ml. normal saline. The effects of these two methods of sensitization were indistinguishable: heat-killed virulent bacilli were used in Exp. 1, 2 and 3 (Table) and B.C.G. in Exp. 4. Sensitivity to tuberculin was estimated by the method of Long and Miles (1950).

Erythrogenic toxin (Dochez N.Y.5 Strain, C.N.2, 100,000 skin test doses in 1 ml. normal saline) was injected intramuscularly daily for 21 days. Similar filtrates from other Group A strains, C.N.10, C.N.238, C.N.335, C.N.532, and heat-inactivated toxin controls (100° 4 hr.)

TABLE.—*Effect on Potency Ratios of Standard Doses of Tuberculin in Guinea-pigs of Repeated Injections of Bacterial Toxins.*

Exp. No.	Treatment compared with control.	Potency ratio.	Variance ratio.	*P.*
1	Streptococcal toxin* (X.C.39)	2·24	23·48	<0·001
	Inactivated toxin (X.C.39)	0·95	<1	—
	Filtrates from:			
2	Erysipelas strain† (C.N.532)	3·56	89·25	<0·001
	Wound strain† (C.N.238)	3·94	134·65	<0·001
	Richards strain† (C.N.10)	1·80	24·80	<0·001
	Dochez N.Y.5 (C.N.2) flocculation value 20LF; rabbit skin value 25 units	3·27	87·02	<0·001
	Rheumatic fever strain† (C.N.532)	1·87	30·74	<0·001
3	*Staph. pyogenes* toxin	0·57	17·80	<0·001
	Streptococcal toxin (X.C.39)	2·41	29·21	<0·001
	Cl. septicum toxin	0·69	5·34	<0·05
	Cl. welchii toxin	0·90	<1	—
	Shig. shigae toxin	1·09	<1	—
	C. diphtheriae toxin	1·08	<1	—
4	Cortisone acetate	0·34	63·41	<0·001
	Cortisone acetate‡ + streptococcal toxin	0·65	16·42	<0·001

* Prevented by previous injection of scarlatinal antitoxin.

† Failed to give any reaction by flocculation or rabbit skin method, and were negative even in a blend test with standard toxin.

‡ Compared with toxin-treated controls.

were diluted irrespective of their erythrogenic toxin content in the same ratio as filtrate C.N.2 and administered in the same way. (The index figures are those of the Wellcome Research Laboratories.)

The non-streptococcal toxins were diluted to produce lesions of comparable size to those produced by C.N.2 when injected intradermally into guinea-pigs. Dilutions with normal saline were made immediately before injection. The dose was slightly misjudged with diphtheria toxin, for guinea-pigs so treated lost weight. The remainder treated with other toxins gained weight as rapidly as the controls. Toxaemia of comparable severity may therefore be assumed to have been produced in all groups of animals except those treated with diphtheria toxin.

As in previous work, the diameter of the tuberculin lesions after 24 hr. was found to be proportional to the logarithm of the dose of tuberculin; and it was possible to estimate the degree of sensitivity by the relative positions of the dosage-response lines fitted to the mean lesion diameters plotted against the logarithm of the dose. Changes in sensitivity are measured in terms of the change in potency of standard doses of tuberculin compared with that in control animals. Thus, a potency ratio of 0·25 indicates a 4-fold decrease, and of 4·00 a 4-fold increase in sensitivity. The probability that ratios differing from unity indicate a significant change in sensitivity is expressed by the value of P in the tables. Ratios for which P is 0·05 or less are considered significant.

RESULTS.

Streptococcal toxins.

Animals injected daily with streptococcal toxin (C.N.2) for 21 days showed a significant (3-fold) increase in sensitivity to tuberculin. Heat-inactivated (100° 4 hr.) toxin was without effect (Exp. 1). Scarlatinal antitoxin injected 24 hr. before the toxin prevented an increase in hypersensitivity.

A single injection of toxin (C.N.2) was without effect, but guinea-pigs injected daily with toxin showed increased sensitivity to tuberculin after one week, and

maintained this increase until the fourth week, after which sensitivity of both groups merged until they did not differ significantly (Fig.). The peak of sensitivity was reached at the third week. Each point on the graph represents the mean of lesion diameters produced by the intradermal injection of 32 and 320 International Units of Old Tuberculin in 0·2 ml. normal saline, into groups of 5 guinea-pigs; degrees of sensitivity are remarkably constant considering that a different group of animals was tested each week, seventy guinea-pigs in all being used.

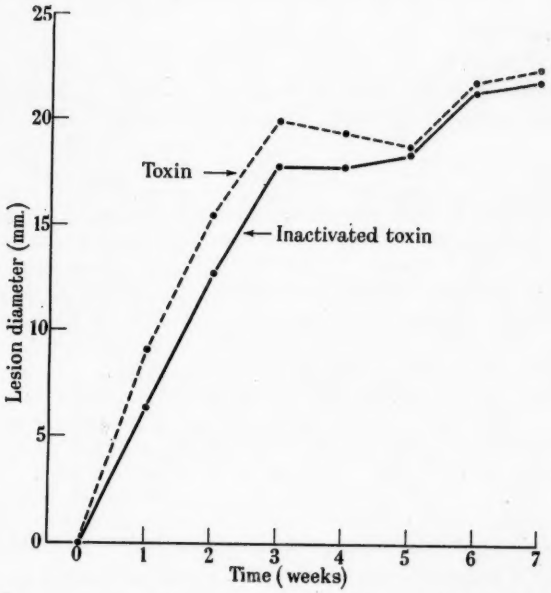

FIGURE.—Susceptibility to tuberculin of skin of sensitized guinea-pigs receiving daily injections of toxin and inactivated toxin.

Filtrates from strains C.N.2, C.N.10, C.N.238, C.N.335, and C.N.532 of haemolytic streptococci all increased sensitivity significantly (Exp. 2). There was no correlation between power to produce hypersensitivity and the erythrogenic toxin content of the filtrate. Streptococcal toxin does not increase the rate of development of the tuberculin response in sensitized guinea-pigs.

Non-streptococcal toxins.

Of six exotoxins tested in Exp. 3, all being injected daily for 21 days, only streptococcal toxin increased sensitivity; staphylococcal toxin, and a filtrate from *Clostridium septicum* significantly depressed sensitivity (Exp. 3). Toxins from *Clostridium welchii*, *Shigella shigae* and *Corynebacterium diphtheriae* were without significant effect.

Thyroid activity.

Thyroid activity was estimated by means of radioactive iodine (see, *e.g.*, Gross and Pitt-Rivers, 1952). The only positive finding was that animals treated with diphtheria toxin had small and less active thyroids than the rest of the group which did not differ significantly from the controls. Repeated experiments were carried out with streptococcal toxin because it was thought possible that this toxin might cause thyroid hyperactivity associated with hypersensitivity to tuberculin (Long and Miles, 1950; Long, Miles and Perry, 1951), but no significant increase in thyroid activity was observed.

Cortisone desensitization.

A single subcutaneous injection of cortisone acetate (5 mg./kg.) proved to have approximately half the desensitizing power in animals treated with streptococcal toxin that it had in animals not so treated (Exp. 4).

DISCUSSION.

Guinea-pigs injected with streptococcal toxin showed a significant increase in sensitivity to tuberculin. This effect was independent of strain or of capacity to produce erythrogenic toxin. It could not be attributed to a non-specific general toxaemia for other exotoxins failed to increase sensitivity even when, as in animals injected with diphtheria toxin, the dose of toxin induced loss of weight. It is noteworthy that the presence of hyaluronidase in toxin from *Cl. welchii* did not apparently influence hypersensitivity.

Because of the long-standing belief that rheumatic fever is associated with hyperthyroidism, repeated but unsuccessful attempts were made to show that streptococcal toxin stimulated thyroid activity and so induced hypersensitivity. It seems, however, more likely that the toxin interferes with cortisone desensitization; whether or not it does so by causing an endocrine disbalance remains to be proved.

The hypothesis that the haemolytic streptococcus initiates rheumatic fever by establishing a low-grade focus of infection associated with hypersensitivity of the tuberculin type to streptococcal allergens, that mesenchymal cells already damaged by streptococcal toxin show heightened susceptibility to streptococcal allergens and increased resistance to desensitization by naturally produced cortisone, is in keeping with clinical and experimental evidence relating haemolytic streptococci to rheumatic fever.

SUMMARY.

Using the response to intradermal tuberculin as a measure of allergic hypersensitivity in albino guinea-pigs sensitized with B.C.G. or with dead virulent tubercle bacilli, it was found that:

1. Group A haemolytic streptococci contain a heat-labile antigen which increases sensitivity to tuberculin: the rate of development of the tuberculin reaction is not affected. The effect is prevented by administering scarlatinal antitoxin before the antigen. When filtrates containing this antigen are injected daily, the peak of hypersensitivity is reached in three weeks. They have no other detectable effect, weight being gained normally.

2. Hypersensitivity in toxin-treated guinea-pigs is less susceptible to cortisone desensitization than that in untreated.

3. The exotoxins of five other species of bacteria have no such effect.

We are indebted to Dr. Knud Tolderlund of the State Serum Institute, Copenhagen, for the B.C.G.; to Dr. A. E. Francis of the Wellcome Research Laboratories, Beckenham, for advice, and for selecting different strains of haemolytic streptococci and making all the streptococcal filtrates for us; to Miss M. V. Mussett for statistical analyses, not only on the experiments described here but also on confirmatory experiments on which our conclusions are based.

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GASTROINTESTINAL FUNCTION IN EXPERIMENTAL HEPATIC INSUFFICIENCY.

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It is well known that anorexia, nausea and vomiting commonly occur in viral hepatitis and to the patient these are usually the most distressing features of the disease. It has sometimes been supposed that these symptoms are due to an associated gastroduodenitis and claims have been made that gastroscopic, radiological and secretory changes indicative of this are demonstrable in some cases of viral hepatitis (Wichels and Brinck, 1933; Knight and Cogwell, 1945; Havens Kushlan and Green, 1947). More extensive and more recent studies of the stomach by gastroscopy and gastric biopsy have failed to distinguish any significant deviations from normal in such patients (Bank and Dixon, 1946; Palmer, 1951) and the cause of the gastrointestinal symptoms of acute hepatitis remains obscure. In the experiments to be described rats were subjected to operative procedure designed to produce acute hepatic insufficiency uncomplicated by damage to other tissues, and the effects of these procedures on gastrointestinal function were studied.

METHODS.

Male rats of the Wistar strain weighing 200–350 g. were used. They were fed for two days pre-operatively on a synthetic diet of corn starch and casein with salts and vitamin supplements. After operation they were fed individually and each animal was offered 15 g. of the same diet twice a day and the amount actually eaten was recorded.

Barium meals.—At operation 4 ml. of a non-flocculating suspension of barium sulphate was introduced into the stomach through a gum-elastic catheter and radiographs were taken approximately 24 hr. after operation. In some animals further films were taken after 48 and 72 hr.

Hepatic necrosis was produced by injecting approximately 0·03 ml. of carbon tetrachloride into a branch of the mesenteric vein at operation. This dose caused a post-operative mortality of nearly 50 per cent and depressed the prothrombin index to about 50 per cent of normal. The control rats were subjected to an identical procedure except that physiological saline was injected into the mesenteric vein. The technique is described more fully elsewhere (Reynell, 1953) and was designed to produce severe hepatic necrosis without significant damage to other tissues.

Subtotal hepatectomy.—Excision of the median and left lateral lobes involves the removal of about 65 per cent of the liver substance, but the animal is not much disturbed by this procedure and a more radical operation is necessary in order to produce symptoms of hepatic insufficiency. In these experiments the median and left lateral lobes were excised and the anterior lobule of the right lobe was removed also. This operation removed some 75 per cent of liver substance, carried a mortality of just under 50 per cent and lowered the prothrombin index to about 50 per cent of normal.

Division of the bile duct.—In two animals the bile duct was doubly ligated above the entry of the pancreatic ducts and then divided between the ligatures. These animals developed

jaundice and hypoprothrombinaemia and excreted bile in the urine, thus establishing the efficacy of the operation.

Abdominal vagotomy was carried out in three animals by dividing both vagi just below the diaphragm. The efficacy of the operation was established by the discovery of gastric distension when the animals were killed 10 days later.

Hexamethonium bromide in a dose of 40 mg./kg. body weight every 6 hr. for 24 hr. was given subcutaneously to four animals. This is more than one-third of the $L.D._{50}$ (Zaimis, personal communication).

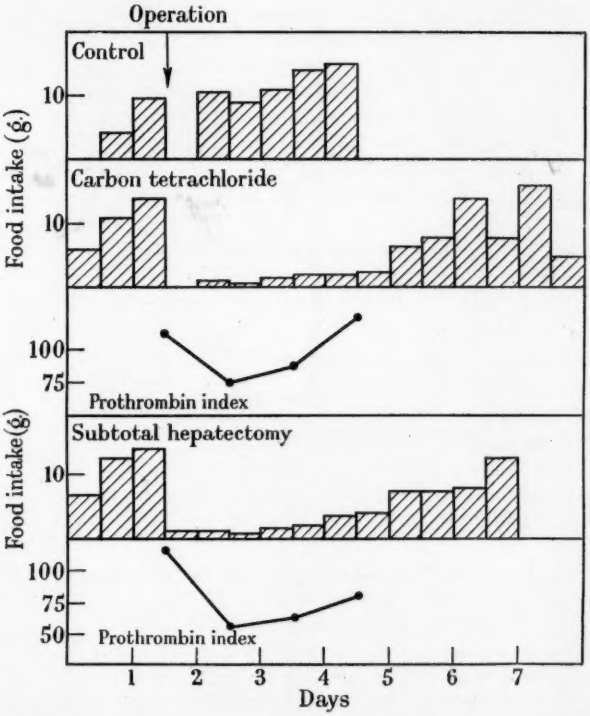


FIG. 1.—Examples of the prolonged anorexia seen in animals given 0·03 ml. carbon tetrachloride into the mesenteric vein or subjected to subtotal hepatectomy, compared with a control animal subjected to a sham operation.

RESULTS.

Effect on appetite.—In control animals subjected to sham operations, appetite remained unimpaired and the animals continued to eat more than 15 g. of food a day after the operation. In animals subjected to subtotal hepatectomy and in those with hepatic necrosis, the appetite was grossly impaired for about four days after operation (Fig. 1). The duration and intensity of the anorexia was approximately proportional to the impairment of liver function as assessed by the prothrombin index, and full appetite was not usually regained until after the prothrombin index had returned to normal.

Effect on gastrointestinal motility.—In six control animals subjected to sham operations the barium had always reached the caecum and colon after 24 hr. (Fig. 2*a*) and none was seen in stomach or small intestine.

In connection with another investigation, serial radiographs have been taken after introducing barium into the stomachs of 24 normal rats of the same strain. In none of these animals was there any barium in the stomach or small intestine after 24 hr., and in only two rats was any seen in the stomach at 6 hr.

In all of 13 animals with hepatic necrosis a large amount of barium was still visible in the stomach and small intestine after 24 hr. (Fig. 2*b*) and it was only rarely that much had reached the caecum. In some animals barium remained in the stomach for more than 72 hr. In the 8 animals with subtotal hepatectomy the effects were similar though rather less striking and less prolonged. Barium was sometimes seen in the stomach 24 hr. after operation, but most of it was seen in the small intestine (Fig. 2*c*) and little had reached the caecum.

In animals subjected to division of the bile duct, gastrointestinal motility was not appreciably disturbed, and after 24 hr. all the barium was in the caecum and colon.

Of the animals subjected to vagotomy and of those given hexamethonium bromide, one vagotomised rat had a little barium in the stomach, which may have been due to coprophagy, but in the others there was no barium in stomach or small intestine at 24 hr.

DISCUSSION.

Subtotal hepatectomy and the injection of carbon tetrachloride into the mesenteric vein were intended to produce an acute reduction in the amount of functioning liver tissue without causing damage to other tissues, and both procedures resulted in a marked anorexia and a profound reduction in the effective motility of the stomach and small intestine. In animals with necrotic livers the changes were rather more striking than in those subjected to surgical removal of liver substance.

The mechanism by which these changes are brought about is not clear. It is unlikely to be a nervous reflex as in some animals the changes persisted for more than 72 hr., and procedures calculated to disturb the autonomic regulation of bowel motility (vagotomy or the injection of a ganglion-blocking substance) failed to produce similar changes. It is not due to a reduction in bile secretion since a complete division of the bile duct did not appreciably delay the passage of barium through the bowel and caused only a slight diminution in appetite. It is more likely to be due to some humoral process—a toxic substance normally inactivated by the liver, the lack of some substance secreted by a healthy liver or some more indirect mechanism. It may at least be concluded that the functional integrity of

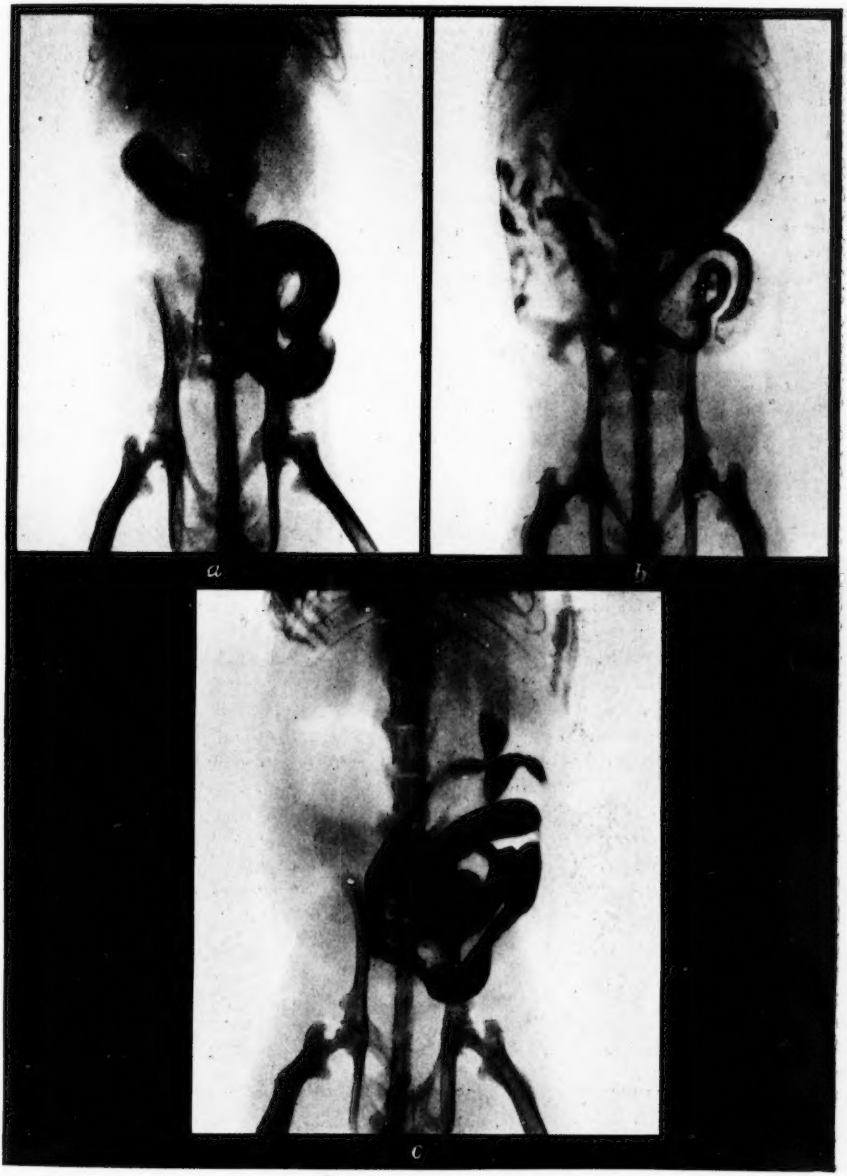
DESCRIPTION OF PLATES.

FIG. 2.—Radiographs taken 24 hours after the injection of 4 ml. of barium sulphate suspension into the stomach.

a. Control rats subjected to sham operation. The contrast medium is seen in caecum and colon only.

b. Rat given 0·03 ml. carbon tetrachloride into mesenteric vein. The contrast medium is seen in stomach and small intestine only.

c. Rat subjected to removal of 75 per cent of liver. The contrast medium is confined to the small intestine.



Reynell.

the gastrointestinal tract of the rat is dependent upon that of the liver, and it is not necessary to postulate a " gastroduodenitis " in order to explain the gastrointestinal disturbances observed in acute hepatitis.

SUMMARY.

Acute hepatic insufficiency uncomplicated by damage to other tissues has been produced in rats both by the injection of a small quantity of carbon tetrachloride into the mesenteric vein and by subtotal hepatectomy.

In both groups of animals there was a profound anorexia lasting for several days. Barium injected into the stomach at operation remained in the stomach and small intestine for more than 24 hr.; this change was most striking in the animals with hepatic necrosis, in which barium was sometimes seen in the stomach after 72 hr. These changes could not be reproduced by division of the bile duct, by abdominal vagotomy or by injections of hexamethonium bromide.

I am grateful to Professor L. J. Witts, in whose department this work was done, to Dr. G. Ardran of the Nuffield Institute for Medical Research for granting radiological facilities, and to Mr. M. S. Tuckey and Miss Shirley Thomas for technical assistance.

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SURVEY OF PAPERS.

MARGARET H. D. SMITH and KUN find that the intact chorio-allantoic membrane of the chick embryo shows increased O_2 uptake and lactic acid production after infection with each of seven viruses; in homogenates of these infected membranes an increase of zymohexase activity was found. These observations were correlated with the histological changes seen at different intervals after infection. Similar changes in enzyme activity were not detected after inoculation with heat-inactivated viruses, and after the production of sterile inflammation by means of Celite were also lacking or of different magnitude. Purified preparations of infective myxoma virus failed to show such enzyme activities (p. 1).

HATA and MITOMI have examined fractions of diphtheria antitoxin prepared by salting out with high and low concentrations of ammonium sulphate. The avidity of these fractions for diphtheria toxins has been shown to differ, and they conclude that salting out is not a satisfactory way of preparing an ideal antiserum (p. 11).

MARGUERITE O'L. CROWE and AMY WALKER have isolated from extracts of virulent *Myco. tuberculosis* of human type a pigment of which various physical and chemical properties appear to identify it as xanthopterin (p. 18).

WILKINS reports a study of the formation of antibacterial substances by a further series of 100 *Basidiomycetes* (p. 28).

HOET, BLOMFIELD and COOMBS have compared the "absorbability" of the haemolytic complements in guinea-pig, human and rabbit sera using antibody from various animal species against mallein. Although some antisera were anti-complementary to normal rabbit serum, complement from this source was shown to be the most sensitive of the three to specific fixation (p. 32).

HEWITT has studied the behaviour of fluids injected subcutaneously by direct observation in reflected skin flaps. Any particulate material in the fluid remained concentrated near the needle point, although the fluid itself distended a much larger volume of connective tissue. This observation accounts for, among other things, the fact that when a tumour is transplanted by injecting a cell suspension the fresh growth arises from a single focus (p. 35).

PEPLER and BRANDT compared the action of elastase and chondro-sulphatase on aorta and costal cartilage and found that the actions of the two enzymes were identical. They suggest that elastin is rendered soluble first by removal of free acid mucopolysaccharides and subsequently by solution of the elastic material itself. They also found that prior staining of elastin inhibits the action of elastase (p. 41).

ANANTHANARAYAN, in a study of the antigenic relationship of influenza viruses with sera produced in guinea-pigs, observed two unusual examples of the pro-zone phenomenon in the complement fixation reaction. The responsible substances in both serum samples were removed by ether treatment, but one survived storage or freezing and thawing and the other did not (p. 46).

HOLT and DEIRDRE M. YATES have found that the concentration of silica and the pH influence the rate of polymerization into polysilicic acid *in vivo*. The degree of polymerization was estimated by comparing the silicon content of the kidney and liver using a tracer technique after intraperitoneal injection of rats with radioactive sodium silicate. They discuss the part played by polymerization in the genesis of fibrous tissue in silicosis (p. 52).

FAZEKAS de ST.GROTH and DORIS M. GRAHAM have further studied the production of incomplete influenza virus particles following heavy allantoic inoculation in eggs. Ten influenza virus strains of different types were tested using inocula of two different magnitudes, haemagglutinating and infective units being titrated after a suitable interval. The ten strains varied greatly in capacity to produce incomplete virus, some forming much and others apparently none, although its formation in small amounts by these strains was proved by serial passage of undiluted inocula. The hypothesis of von Magnus accounting for incomplete virus production is disputed, and another proposed (p. 60).

ALBERT, HAMPTON, SELBIE and ROSEMARY D. SIMON have set out to test the hypothesis that 8-hydroxyquinoline (oxine) acts within the bacterial cell. They have examined the antibacterial activity of analogues of oxine, and have shown that this action is lost when the oil/water partition coefficient is reduced, and is restored when it is raised. Assuming that the ease with which a substance penetrates a cell membrane is proportional to its lipophilic nature, and that this nature can be measured in terms of the oil/water partition coefficient, they conclude that their findings support the hypothesis that oxine acts within the cell (p. 75).

ROSLYN C. GREY and LONG have shown that sensitivity to tuberculin in guinea-pigs is increased by injections of haemolytic streptococcal culture filtrates: this effect is obtained whether the streptococcus forms erythrogenic toxin or not, and it is neutralised by scarlatinal antitoxin. Other bacterial exotoxins did not have this effect. The findings are discussed in relation to the mechanism of production of rheumatic fever (p. 85).

REYNELL has shown radiologically in rats that in acute hepatic insufficiency produced by either subtotal hepatectomy or carbon tetrachloride poisoning, the rate of passage of food from the stomach onwards is greatly reduced. Such animals consumed little food for several days. He suggests that anorexia and nausea in viral hepatitis may be a direct consequence of hepatic insufficiency and not due to a hypothetical gastroduodenitis (p. 92).

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